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14. ABSTRACT

A summary is presented of research performed on the DoD Idea Award to investigate mixed-lineage kinase 3 in breast cancer invasion and metastasis. Three aims were pursued. The first aim was to determine the signaling basis of the novel migratory role of MLK3 in breast cancer cells. The second was to determine whether the impact of MLK3 on migration and invasive phenotype extends to a broad range of human breast cancer cell lines. The third aim was to evaluate MLK3 as a potential therapeutic target for metastatic breast cancer using in vivo models. The longterm goal of these studies was to provide a foundation for determining whether MLK3 is a viable therapeutic target for invasive/metastatic breast cancer and to rigorously determine the signaling pathways through which MLK3 operates in breast cancer cells.

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INTRODUCTION:

There is a profound need for new effective therapies for treating invasive and metastatic breast cancer. The overall hypothesis of this research is that signaling pathways through mixed-lineage kinase 3 (MLK3) control breast cancer cell migration, invasion, and metastasis. Our data indicate that active MLK3 is required for migration of invasive human breast cancer cells and that active MLK3 promotes migration of a noninvasive breast cancer cell line. This presents the exciting possibility that existing MLK inhibitors might be effective therapies for patients with invasive and metastatic breast cancer, or for prevention of invasive and/or metastatic breast cancer. Identification of the components of migratory and metastatic signaling pathways impacted by MLK3 in a panel of metastatic and non-metastatic human breast cancer cell lines would be useful in pre-identifying tumors that are predicted to respond to MLK inhibitors. The scope of this project includes identifying MLK3-mediated signaling pathways in breast cancer cell lines, determining whether MLK3 is required for in vitro migration and invasion and evaluating whether Mlk3-silencing and/or inhibitors affect metastasis in mouse xenograft models of human breast cancer.

BODY:

Task 1. To identify MLK3-dependent signaling alterations in breast cancer cell lines

The impact of MLK3 on MAPK signaling in mammary epithelial and breast cancer cell lines was investigated. MLK3 can activate multiple MAPK pathways including the ERK, p38, and JNK pathways. Our data,(see appended publication, Oncogene, Chen et al., 2010) (1), demonstrate that MLK3 signaling to JNK is critical for breast cancer migration and invasion. JNK is well-known to activate the transcription factor AP-1, in part through JNK-mediated phosphorylation of c-jun. Several AP-1 regulated genes have been associated with breast cancer invasion. For our studies we established an inducible expression system for MLK3 in MCF10A human mammary epithelial cells. We found that induction of active MLK3 promotes migration and invasion of MCF10A cells, which can be blocked by pharmacological inhibitors of JNK and MLKs. Active MLK3 signaling in MCF10A induces the expression of AP-1-regulated invasion genes, including c-jun, fra-1, and vimentin. To rigorously determine the contribution of AP-1 to MLK3-mediated invasion, we expressed a dominant negative inhibitor of the AP-1, known as Tam67, in the MLK3-inducible MCF10A cells and showed that it blocked MLK3-induced invasion and expression of the AP-1 invasion gene, fra-1.

Paxillin is an adaptor protein that organizes focal adhesion complexes that mediate cell adhesion. Turnover of focal adhesions is important for rapid cell migration. In response to epidermal growth factor, JNK phosphorylates paxillin on Ser 178 in invasive MDA-MB-231 cells, and promotes migration (2); (3). Pharmacological inhibition of JNK, or expression of a paxillin variant that cannot be phosphorylated by JNK, blocks migration. This phosphorylation event is critical for focal adhesion turnover, leading to rapid cellular migration. We showed that JNK is critical for MLK3 to mediate cell

migration and invasion. Given the concordance of these findings with our preliminary data in MDA-MB-231 migration, we hypothesized that MLK3 might promote JNK-mediated phosphorylation of paxillin to promote cell migration in response to extracellular factors that promote motility. Our data and findings have been published in Cancer Research (appended to this report Chen and Gallo, 2012) (4). To determine whether MLK3 regulates paxillin phosphorylation, we used immortalized mammary epithelial cells MCF10A, which we had engineered to inducibly express MLK3 (MCF10A-iMLK3 cells). Induced expression of MLK3 robustly promotes paxillin phosphorylation at Ser178. Inhibition of MLK or JNK activity, but not of ERK or p38, blocks this paxillin phosphorylation. In invasive human breast cancer MDA-MB-231 cells, forced expression of wildtype MLK3 but not a kinase dead mutant of MLK3 K144R increases paxillin phosphorylation at Ser178. To confirm that JNK is required for paxillin phosphorylation at Ser178, endogenous JNK1,2 was silenced by siRNA. JNK silencing decreases paxillin phosphorylation at Ser178. Taken together, these data demonstrate that active MLK3 is sufficient to induce JNK-mediated paxillin phosphorylation. To determine whether endogenous MLK3 is required for paxillin phosphorylation, MDA-MB- 231 cells stably expressing a control vector or shMlk3 were serum deprived and then retreated with serum and phosphorylation of Ser178 of paxillin was assessed. MLK3 silencing attenuates serum-induced phosphorylation of Ser 178 on paxillin. Inhibition of MLK using the pan-MLK inhibitor CEP-1347 reduces paxillin phosphorylation in response to CXCL12 or hepatocyte growth factor, two factors that promote breast cancer cell migration and that have been implicated in breast cancer metastasis. In addition, silencing of MLK3 markedly blocks phosphorylation of Ser178 in BT549 cells in response to CXCL12 or HGF. This result was confirmed using a second MLK3 siRNA. MLK inhibition also impairs paxillin phosphorylation in BT549 cells in response to CXCL12 or HGF. Our data demonstrate that endogenous active MLK3 kinase is required for paxillin phosphorylation in response to CXCL12 or HGF and implicate MLK3 in critical signaling pathways associated with breast cancer metastasis.

In summary, we have identified the MLK3-JNK signaling pathway as a critical mediator of breast cancer cell migration and invasion. Our data support a critical role for the MLK3-JNK-AP-1 signaling axis in induction of invasion genes. We also have identified a novel MLK3-JNK-paxillin signaling axis that contributes to focal adhesion turnover and suppression of Rho activity in migrating breast cancer cells.

Task 2. To determine whether the impact of MLK3 on migration and invasive phenotypes extends to a broad range of human breast cancer cell lines

We used multiple approaches to test the impact of MLK3 signaling on breast cancer migration and invasive phenotypes. MLK3 silencing, as well as an MLK inhibitor, CEP-11004, is able to block migration of the highly invasive MDA-MB-231 cells. Our findings were extended to additional human breast cancer cell lines, including MDA-MB-435 and Hs578t. In complementary experiments we demonstrated that induced expression of active MLK3 is sufficient to promote migration of weakly invasive MCF-7 cells as well as of the immortalized mammary epithelial MCF10A cells. The impact of MLK3 silencing in the triple negative human breast cancer cell line, BT549, and MDA-MB-231 cells has been investigated in detail to better understand the connection between MLK3 signaling

and the role of MLK3 in breast cancer cell invasion and migration. We sought to determine whether MLK3 impacts migration and/or invasion in response to key extracellular factors implicated in human breast cancer metastasis. In particular we demonstrated that the prometastatic chemokine (5), CXCL12, promotes MLK3-dependent paxillin phosphorylation. Though MDA-MB-231 is a commonly used invasive cell line for breast cancer studies, we extended these studies to BT549 cells. Silencing of MLK3 in BT549 cells also profoundly blocks migration and invasion through Matrigel in response to the prometastatic chemokine CXCL12, analogous to our findings in MDA-MB-231 cells.

We also used a 3D Matrigel culture system to inducibly express MLK3 in preformed mammary acini of MCF10A cells. These data are published in the appended Oncogene manuscript (Chen et al, 2010) (1). In this structurally appropriate environment, induction of MLK3 promotes proliferation and luminal filling and modest disruption of polarity. Thus we have demonstrated the potential of MLK3 in multiple aspects of the malignant phenotype including proliferation, migration, and antiapoptotic signaling. Our findings highlight the importance of the structural environment (2D versus 3D culture) in assessing oncogenic phenotypes. There is a growing interest in the use of 3D culture systems as tools for screening anticancer drugs, since the ability of pharmacological inhibitors to revert malignant phenotypes may reflect therapeutic efficacy (6). Hence the ability of the MLK3 inhibitor, CEP-11004, or MLK3 silencing to alter the malignant phenotype of the invasive breast cancer cell lines grown in 3D Matrigel cultures was determined. Silencing of MLK3 markedly disrupts the malignant phenotype in invasive MDA-MB-231 cells grown in 3D Matrigel. Furthermore an MLK inhibitor, CEP-11004 drastically reduces the number of invasive structures of MDA-MB-231 cells in 3D culture.

Task 3. To evaluate MLK3 as a potential therapeutic target for metastatic breast cancer using *in vivo* models

As the first step toward evaluating MLK3 as a potential therapeutic target for metastatic breast cancer, we used a mouse xenograft model. MDA-MB-231 cells orthotopically transplanted into immunodeficient mice will form tumors that metastasize to the lungs. MDA-MB-231 cells stably expressing a control or *mlk3* shRNA vector were orthotopically implanted into the mouse mammary gland. After 7 weeks, the mice were sacrificed and the primary tumors were excised and measured. The lungs were harvested and metastases in lung sections were visualized using a human specific antibody directed against CD44. Silencing of mlk3 has no significant impact on size of the primary tumor. Immunoblotting of tumor cell lysates show that mlk3 silencing was maintained over the course of the experiment. Silencing of mlk3 blocked formation of lung micrometastases. These results demonstrate that MLK3 plays a critical role in the metastatic process.

Another goal is to determine whether MLK inhibitors would be effective in preventing formation of metastases (7). In order to follow the impact of an MLK inhibitor on MDA-MB-231 metastasis our approach was to use bioluminescence imaging using the IVIS

imaging system. We opted to use a commercially available MDA-MB-231 cell line that stably expresses RFP (tomato fluorescent protein) and luciferase. To assess the tumorigenic capacity of MDA-MB-231-luc2-tdTomato, different routes of administration were tested: subcutaneous (SC), mammary fat pad (MFP), and intraperitoneal (IP). All experiments were performed in accordance with MSU IUCAC-approved protocols. Luminescence imaging was performed after IP injection of D-luciferin at a final concentration of 150 mg/kg (8). We were able to successfully establish a luciferase imaging protocol. However, the commercially purchased labeled MDA-MB-231 cell line was poorly tumorigenic even after 3 months post transplantation and no metastases were detected using this cell line. We obtained another MDA-MB-231 cell line from Caliper/Perkin Elmer that was derived from a lymph node metastasis and is reported to form lung metastases. MDA-MB-231-luc-D3H2LN-bearing mice exhibited detectable metastases 6 weeks post transplantation, but at this time primary tumors were already quite large. This system could work for our studies but may require removal of primary tumor. An alternative system was also investigated for these studies. 4T1 are a mouse basal-like mammary tumor cell line. Using orthotopic transplantation of 4T1-luc2 into mammary gland of athymic nude mice, metastases were detectable within 15 days. Thus this system, though it is a mouse rather than human line, would provide a robust system for detecting the impact of the MLK inhibitor on 4T1 metastases.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that MLK3 silencing prevents migration of multiple human breast cancer cell lines.
- Showed that induced expression of MLK3 in immortalized mammary epithelial cells promotes migration.
- Determined that the MAPK signaling pathway that is predominantly required for migration across a range of human breast cancer and MLK3-expressing mammary epithelial cells is the JNK pathway.
- Demonstrated that MLK3 expression promotes invasion of MCF10A mammary epithelial cells.
- Showed that MLK3 induces invasion associated genes, including vimentin, fra-1, and c-jun.
- Determined that MLK3-induced invasion requires activity of the transcription factor AP-1.
- Showed that MLK3 induces phosphorylation of paxillin, an event required for focal ahesion turnover and rapid cell migration.
- Determined that MLK3-induced phosphorylation of paxillin requires JNK activity.
- Demonstrated that MLK3 silencing blocks Ser 178 phosphorylation of paxillin in response to serum, chemokine CXCL12, and growth factor HGF in MDA-MB-231 cells.
- Showed that MLK3 silencing blocks paxillin phosphorylation in triple-negative BT549 breast cancer cells.
- Determined that an MLK inhibitor blocks chemokine and growth factor-induced

phosphorylation of paxillin in BT549 cells.

- Showed that MLK3 silencing blocks migration and chemoinvasion of the triple negative BT549 cells in response to both serum and to the prometastatic chemokine, CXCL12.
- Determined that MLK3 silencing disrupts the malignant phenotype of MDA-MB-231 cells in 3D Matrigel culture.
- Demonstrated that an MLK inhibitor blocks the malignant phenotype of MDA-MB-231 cells in 3D Matrigel culture.
- Showed that silencing of MLK3 does not impact primary tumor size but prevents formation of lung metastases using MDA-MB-231 cells in a mouse xenograft model.
- Provided additional findings involving regulation of phosphorylation of paxillin by MLK3
- Established a protocol for luciferase activity in mice for in vivo imaging.
- Compared of routes of administration of tumor cells and resulting formation of primary tumor and/or metastases.
- Evaluated the relative metastatic capacity of multiple MDA-MB-231 sublines and 4T1 breast cancer cells.

REPORTABLE OUTCOMES:

Publications:

- Chen J, Miller EM, Gallo KA. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. Oncogene. 2010 Aug 5;29(31):4399-411
- Chen J and Gallo KA. MLK3 regulates paxillin phosphorylation in chemokine-mediated breast cancer cell migration and invasion to drive metastasis Cancer Res. 2012 Aug 15;72(16):4130-40. doi: 10.1158/0008-5472.CAN-12-0655.

Abstracts and presentations:

AACR special meeting-Tumor Invasion and Metastasis, Published abstract and poster presentation by Chotirat Rattanasinchai "MLKs regulate the activities of the small GTPases, Rho and Rac, to drive both single cell and collective cell migration in breast cancer invasion". January 21-23, 2012; San Diego, CA.

Summer Research Opportunity Program (SROP) at MSU (Aug, 2012) and Research Initiative for Scientific Enhancement (RISE) Nov. 1, 2012, "The Effect of MLK Inhibitor on Metastatic Mouse Cancer Cells", Presentation by Lishann Ingham,MSU-SROP undergraduate student, East Lansing, MI

DoD Era of Hope Meeting, Published abstract and poster presentation, "MLK3 signaling in breast cancer invasion and metastasis", Aug. 2-5 2011, Baltimore, MD

MRS-AACR Joint Conference on Metastasis and Tumor Microenvironment, September

Published abstract and poster presentation. “The functional role of mixed lineage kinase 3 in breast cancer metastasis”. Chen J, Miller, EM, and Gallo KA. September 12-15, 2010 Philadelphia, PA.

Gordon Research Conference: Signaling by adhesion receptors. Poster presentation “MLK3 signaling in breast cancer metastasis” July 11-16, 2010 Waterville, Maine.

Midwest Breast Cancer Research Symposium. *The University of Iowa / MERF*. Oral presentation and abstract. “Mixed Lineage Kinase 3 signaling in breast cancer” July 17-19, 2009.

FASEB Summer Research Conference. Protein Kinases and Protein Phosphorylation Poster presentation and abstract.”MLK3 signaling in breast cancer cell migration” July 26-31, 2009.

Cold Spring Harbor Meeting on Phosphorylation, Signaling and Disease. *Cold Spring Harbor Laboratory.*Poster presentation and abstract by Jian Chen, graduate student. “MLK3 signaling in breast cancer metastasis” May, 2009.

Funding applied for based on work supported by this award:
Jian Chen, who is partially supported by this award, was the 2010 awardee of the Barnett Rosenberg Fellowship in Biological Sciences.
A Komen Foundation Pre-proposals was not invited.
A Pardee Foundation proposal was funded.
A DoD Expansion Proposal is currently under review.
NIH RO1 grant in preparation.

Degrees supported by this award:
Jian Chen, a Biochemistry & Molecular Biology graduate student, partially supported by this award; currently a cancer research postdoc in Dr. Blobe’s lab at Duke

Chotirat Rattanasinchai, Cell & Molecular Biology graduate student, recipient of Royal Thai Fellowship, partial support from this award

Xiaoting Wu, Cell & Molecular Biology graduate student, partial support

Employment and Research Opportunities:
Ashley Sample, Biochemistry undergraduate student, attending UChicago grad school in cancer research

Shivani Rami, Human Biology undergraduate student

Jonathan Kasper, Physiology Master’s student; currently in Physiology PhD program

Lishann Ingram, McNair SROP Summer Undergraduate Research Program

Sean Misek, Chemistry undergraduate

CONCLUSIONS

Our data, taken altogether, demonstrate that MLK3 signaling is critical for breast cancer cell migration, invasion, and metastasis. This is an important finding since the requirement for MLK3 catalytic, rather than scaffolding, activity in breast cancer cell migration and invasion indicates that MLK inhibitors might be useful in treating invasive breast cancer. We have shown that MLK3 is critical for migration and invasion in a broad range of breast cancer cell lines, suggesting a fairly general role for MLK3 in these processes. MLK3 signaling through JNK to activate the AP-1 transcription factor is required for MLK3-induced invasion. This implies that an MLK3 gene expression program may drive breast cancer cell migration and invasion. Furthermore we made the novel finding that the prometastatic chemokine CXCL12 signals through MLK3 to JNK to cause migration and invasion of breast cancer cells. The MLK3-dependent CXCL12-induced migration and invasion is accompanied by phosphorylation of the focal adhesion scaffold protein, paxillin on Ser 178. This phosphorylation event is important for regulating focal adhesion dynamics; rapid turnover of focal adhesions is critical for cell migration. Thus we have discovered a novel role for MLK3 in focal adhesion dynamics. Finally we have shown that silencing of MLK3 prevents formation of lung micrometastases in immunodeficient mice after orthotopic transplantation of human breast cancer MDA-MB-231 cells in the mouse mammary gland.

"So what": Our findings support a critical role for MLK3 in breast cancer invasion and/or metastasis. Furthermore, our data indicate that MLK3 catalytic activity is required for regulation of focal adhesion dynamics which, in turn, is critical for rapid cell migration. MLK3 also promotes the expression of at least some invasion-associated gene. We have found that an MLK inhibitor can block breast cancer migration and invasion, as well as MLK3 signaling events we have identified. This new knowledge is important because it supports the concept that MLK inhibitors may be useful in preventing breast cancer invasion and/or metastases.

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Appendices

Chen J, Miller EM, Gallo KA. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. Oncogene. 2010 Aug 5;29(31):4399-411

Chen J and Gallo KA. MLK3 regulates paxillin phosphorylation in chemokine-mediated breast cancer cell migration and invasion to drive metastasis Cancer Res. 2012 Aug 15;72(16):4130-40. doi: 10.1158/0008-5472.CAN-12-0655.

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ORIGINAL ARTICLE

MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells

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The malignant phenotype in breast cancer is driven by aberrant signal transduction pathways. Mixed-lineage kinase-3 (MLK3) is a mammalian mitogen-activated protein kinase kinase kinase (MAP3K) that activates multiple MAPK pathways. Depending on the cellular context, MLK3 has been implicated in apoptosis, proliferation, migration and differentiation. Here we investigated the effect of MLK3 and its signaling to MAPKs in the acquisition of malignancy in breast cancer. We show that MLK3 is highly expressed in breast cancer cells. We provide evidence that MLK3's catalytic activity and signaling to c-jun N-terminal kinase (JNK) is required for migration of highly invasive breast cancer cells and for MLK3-induced migration of mammary epithelial cells. Expression of active MLK3 is sufficient to induce the invasion of mammary epithelial cells, which requires AP-1 activity and is accompanied by the expression of several proteins corresponding to AP-1-regulated invasion genes. To assess MLK3's contribution to the breast cancer malignant phenotype in a more physiological setting, we implemented a strategy to inducibly express active MLK3 in the preformed acini of MCF10A cells grown in 3D Matrigel. Induction of MLK3 expression dramatically increases acinar size and modestly perturbs apicobasal polarity. Remarkably, MLK3 expression induces luminal repopulation and suppresses the expression of the pro-apoptotic protein BimEL, as has been observed in Her2/Neu-expressing acini. Taken together, our data show that MLK3–JNK–AP-1 signaling is critical for breast cancer cell migration and invasion. Our current study uncovers both a proliferative and novel antiapoptotic role for MLK3 in the acquisition of a malignant phenotype in mammary epithelial cells. Thus, MLK3 may be an important therapeutic target for the treatment of invasive breast cancer.
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Keywords: MLK3; JNK; migration; invasion; Bim; AP-1; breast cancer

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Introduction

Invasive ductal carcinoma accounts for 70% of invasive breast cancer. Cancer cell migration is fundamentally required for breast tumor invasion and metastasis. The migratory phenotype afforded to breast cancer cells is thought to be accompanied by a transient epithelial-to-mesenchymal transition in which genes associated with migration and invasion are upregulated. Malignant breast cancer cells must also bypass normal growth arrest, evade apoptosis and gain limitless replicative potential (Hanahan and Weinberg, 2000). These alterations are not always cancer-cell-autonomous. Interactions of breast cancer cells with extracellular matrix components and other cell types clearly influence phenotypic outcome.

Aberrant signal transduction pathways within tumor cells govern the malignant processes. Delineating the signaling pathways that contribute to the acquisition of the malignant phenotype in breast cancer is crucial for developing interventional strategies. Of the numerous proteins that have been implicated in breast cancer, kinases have emerged as excellent therapeutic targets (Zhang *et al*., 2009), as evidenced by the use of the monoclonal antibody, Trastuzumab, and the small-molecule, ATP mimetic, HER-2/epidermal growth factor receptor (EGFR) dual receptor tyrosine kinase inhibitor, lapatinib, in treating many patients with HER-2-positive breast cancer (Nielsen *et al*., 2009).

The mitogen-activated protein kinase (MAPK) pathways respond to diverse extracellular stimuli to regulate cellular processes including proliferation, differentiation, migration, survival and apoptosis (Johnson and Lapadat, 2002). The three best-characterized mammalian MAPKs are extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. MAPKs have numerous cellular substrates, including transcription factors. For instance, JNK activates the heterodimeric transcription factor, AP-1, by phosphorylation of one of its components, c-jun. While the number of MAPK isoforms is rather limited, there are many more mitogen-activated protein kinase kinase kinases (MAP3Ks), suggesting that MAP3Ks may function as key nodes for integrating diverse extracellular stimuli with MAPK signaling. MLK3 is a widely expressed MAP3K that contributes to the activation of multiple MAPK pathways. MLK3 activates JNK and,

in some experimental settings, also the p38 pathway, by phosphorylating the activation segment of the cognate MAP2K (Gallo and Johnson, 2002). MLK3 apparently acts as a scaffold for the MAP3K, B-Raf, that is required for B-Raf-mediated ERK activation (Chadee and Kyriakis, 2004).

MLK3, with a predicted molecular weight of 92 kDa, contains an N-terminal *src*-homology-3 domain, followed by a kinase catalytic domain with similarity to both serine/threonine and tyrosine kinases, leucine zipper regions, a Cdc42/Rac-interactive binding motif and a large C-terminal tail rich in Ser, Thr and Pro residues (Gallo *et al*., 1994; Gallo and Johnson, 2002). We previously discovered that MLK3 is autoinhibited through its *src*-homology-3 domain (Zhang and Gallo, 2001). Activated Cdc42/Rac increases MLK3 catalytic activity (Teramoto *et al*., 1996) through binding to the Cdc42/Rac-interactive binding motif (Bock *et al*., 2000) and promoting activation loop (auto)phosphorylation (Du *et al*., 2005). On the basis of work from our laboratory and by others (Leung and Lassam, 1998; Vacratsis and Gallo, 2000; Nihalani *et al*., 2001), we propose that binding of the small GTPase, Cdc42 or Rac, disrupts *src*-homology-3-mediated autoinhibition, promoting leucine zipper-mediated dimerization and subsequent transphosphorylation within the catalytic domain to yield active MLK3.

Given its potential for diverse signaling, we undertook an investigation of the role of MLK3 in human breast cancer. Using gene silencing, as well as an MLK inhibitor, we show a crucial requirement for MLK3 in the migration of invasive breast cancer cells. In complementary experiments, induced expression of active MLK3 is shown to promote the migration of poorly invasive breast cancer and non-tumorigenic mammary epithelial cells. Our data indicate that MLK3 signaling to JNK is crucial for breast cancer cell migration. In addition, MLK3 induces the expression of c-jun, fos-related antigen-1 (Fra-1) and vimentin, all of which have been associated with epithelial-to-mesenchymal transition and invasive breast cancer. We further show that MLK3 promotes invasion in an AP-1-dependent manner. To mimic how aberrant MLK3 signaling might affect the normal mammary ductal epithelium, active MLK3 was induced in preformed MCF10A mammary acini grown in a three-dimensional (3D) Matrigel culture system. MLK3 induction causes the mammary acini to bypass growth arrest, increase in size and reinitiate luminal filling. Thus, in the physiological context of a 3D Matrigel culture, MLK3 promotes the acquisition of the principal aspects of the malignant phenotype in mammary acini that, in some respects, mimic the pathological findings in ductal carcinoma *in situ* (DCIS) and invasive breast cancer.

Results

MLK3 is required for migration of highly invasive breast cancer cells

Total cellular lysates were generated from a panel of human mammary epithelial and breast cancer cell lines, and analyzed by western blotting using an MLK3-specific antibody. As shown in Figure 1, higher levels of MLK3 protein were observed in the breast cancer cell lines MCF-7, T47D, SK-BR-3 and MDA-MB-231, as compared with that in the non-tumorigenic mammary epithelial cell lines 184B5 and MCF10A. No apparent correlation was found between MLK3 protein levels and steroid hormone receptor status or metastatic potential.

To probe the function(s) of MLK3 in breast cancer, a gene-silencing approach was taken. An Mlk3 short-hairpin

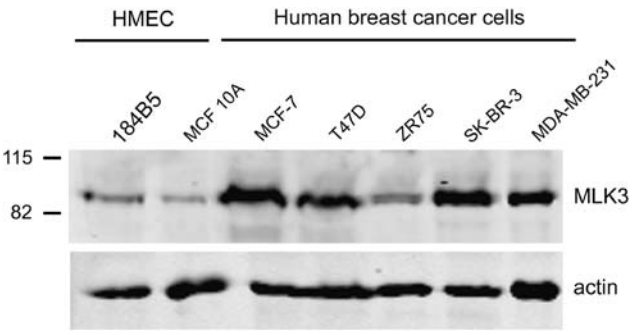
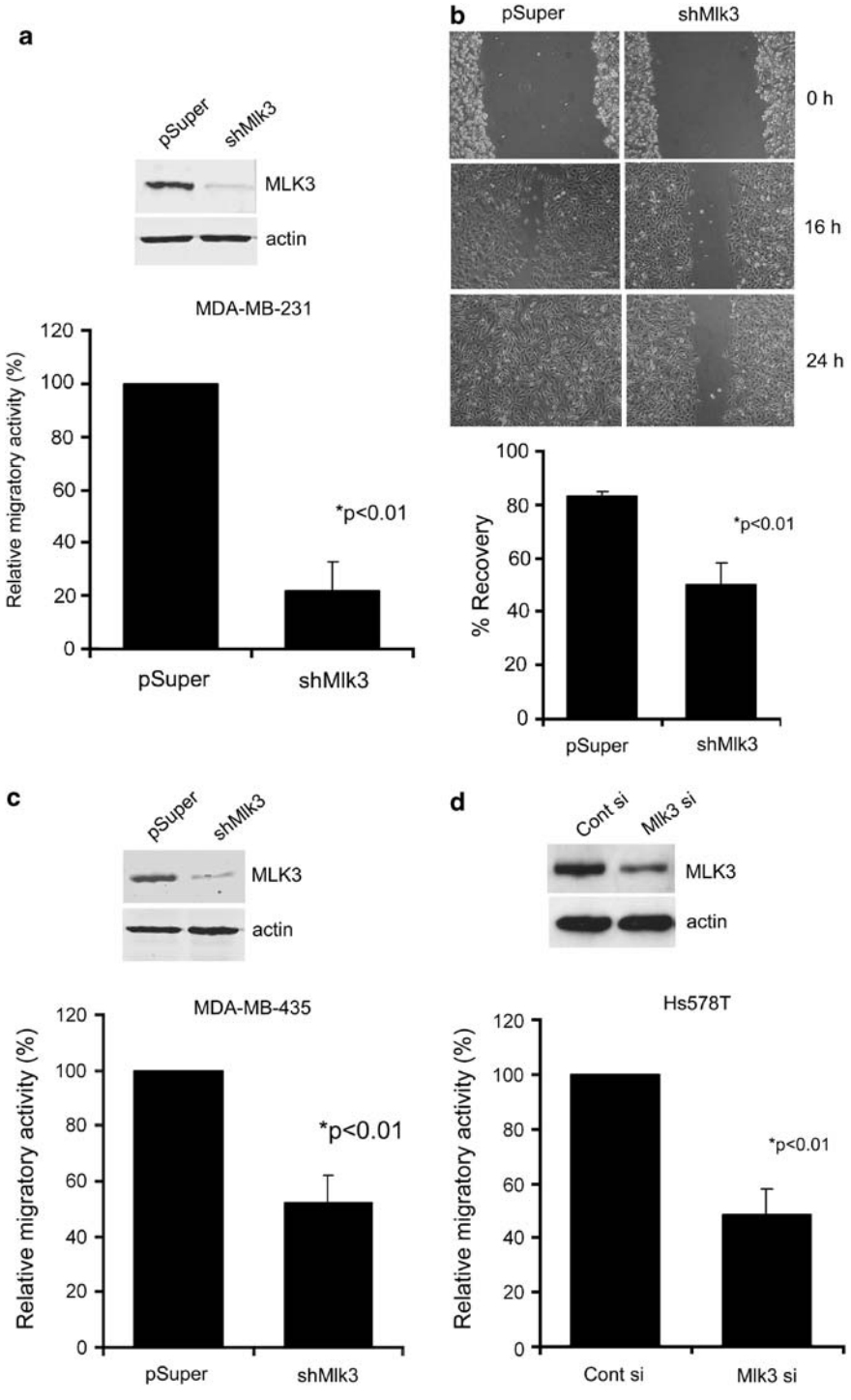


Figure 1 MLK3 levels in human non-tumorigenic mammary epithelial cells and breast cancer cells. Cellular lysates containing 30 μg of total protein from each of the indicated cell lines were subjected to western blot analysis using an MLK3 antibody. Western blot of actin serves as a loading control. The numbers to the left of the MLK3 western blot indicate the mobility of molecular weight markers in kDa. MLK3, mixed-lineage kinase-3.

Figure 2 Silencing of MLK3 markedly reduces the migration of highly invasive breast cancer cells. (**a**) The MLK3 levels in a population of MDA-MB-231 cells stably expressing pSuper or pSuper-shMlk3 were assessed by western blot analysis (upper panel). Western blots of actin are shown as loading controls. MDA-MB-231 control cells or cells depleted of MLK3 were subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 5% serum for 6 h. Triplicate wells were used for each of three independent experiments. The results are expressed relative to the migration of the control pSuper cells (= 100%). Migration was significantly inhibited in MDA-MB-231 cells stably expressing shMlk3 as compared with that in control pSuper-expressing cells. Column: Mean of three experiments; bar: s.e. *P*-value was determined by Student's *t*-test (**P* < 0.01). (**b**) MDA-MB-231 control cells or MDA-MB-231 cells depleted of MLK3 were subjected to a wound-healing assay as described under Materials and methods. Representative photographs at the indicated time points from three independent experiments, each performed in triplicate wells, are shown. Magnification: × 10. The extent of wound recovery was determined as described under Materials and methods. Column: Mean of three experiments. Bar: s.e. (**P* < 0.01). (**c**) MLK3 levels in a population of MDA-MB-435 cells stably expressing pSuper or pSuper-shMlk3 were assessed as described in panel **a** (upper panel). The effect of Mlk3 silencing on the migration of MDA-MB-435 cells was determined as described in panel **a**. Column: Mean of three experiments. Bar: s.e. *P*-value was determined by Student's *t*-test (**P* < 0.01). (**d**) MLK3 levels in Hs578T cells upon transient transfection of control or Mlk3 siRNA for 48 h as described under Materials and methods and in panel **a** (upper panel). The effect of Mlk3 silencing on the migration of Hs578T cells was determined as described in panel **a**. Column: Mean of three experiments. Bar: s.e. *P*-value was determined by Student's *t*-test (**P* < 0.01). MLK3, mixed-lineage kinase-3; siRNA, short interfering RNA.

RNA (shRNA) based on a previously validated short interfering RNA (siRNA) (Chadee and Kyriakis, 2004) was constructed in the pSuper vector and introduced into MDA-MB-231 cells using a retrovirus (Ory *et al*., 1996). Stable populations of MDA-MB-231 cells containing pSuper-Mlk3 shRNA or the control vector pSuper were selected and subjected to proliferation and transwell migration assays. MLK3 knockdown was highly efficient as judged by western blotting (Figure 2a). MLK3 knockdown had no effect on the proliferation of MDA-MB-231 cells in a two-dimensional culture on plastic (Supplementary Figure 1a). However, depletion of MLK3 dramatically reduced the migration of MDA-MB-231 cells by over four-fold in a 6-h transwell assay (Figure 2a). Comparable results were observed upon transient silencing of MLK3 (data not shown). As an alternative approach to study migration, a wound-healing assay was used. As shown in Figure 2b,



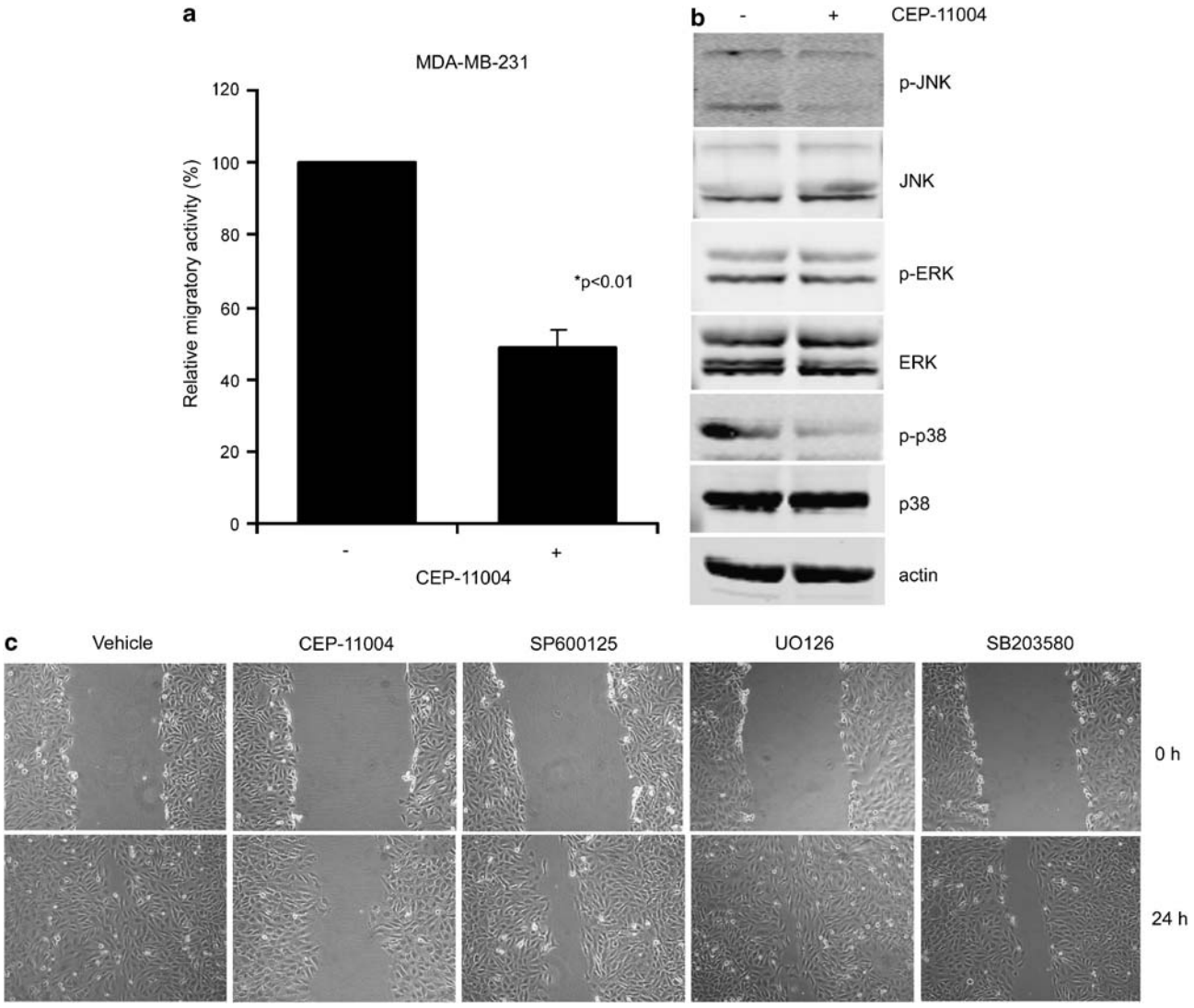


Figure 3 An MLK-selective inhibitor significantly decreases the migration of MDA-MB-231 cells. (**a**) MDA-MB-231 cells were treated 400 nM CEP-11004 or a vehicle (−) for 30 min and subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 5% serum for 6 h. Triplicate wells were used for each of three independent experiments. The results are expressed relative to the migration of the vehicle control (= 100%). Migration was significantly inhibited in MDA-MB-231 cells treated with CEP-11004 as compared with in cells treated with the vehicle. Column: Mean of three experiments. Bar: s.e. *P*-value was determined by Student's *t*-test (**P* < 0.01). (**b**) The effect of CEP-11004 on MAPK activation in MDA-MB-231 cells. MDA-MB-231 cells were treated with a vehicle (−) or 400 nM CEP-11004 for 24 h. Total cellular lysates containing 60 μg of total protein were analyzed by western blotting using the indicated antibodies. (**c**) The effects of MLK and MAPK pathway inhibitors on wound healing in MDA-MB-231 cells were determined as described under Materials and methods and Figure 2b. Immediately after wounding, the medium was replaced with fresh growth medium and the vehicle or the indicated inhibitor (400 nM CEP-11004, 15 μM SP600125, 10 μM U0126 or10 μM SB203580). Representative photographs from three independent experiments, each performed in triplicate wells, at $t = 0$ and at $t = 24$ h are shown. Magnification: × 10. MAPK, mitogen-activated protein kinase; MLK3, mixed-lineage kinase-3; siRNA, short interfering RNA.

MDA-MB-231 cells depleted of MLK3 are significantly impaired in wound recovery measured at 16 h. By 24 h, control MDA-MB-231 cells had completely closed the wound, whereas an obvious wound remained in MLK3-depleted MDA-MB-231 cells. Due to the length of this assay, mitomycin-C was added to rule out any possibility of cell proliferation contributing to the observed phenotype.

To determine whether MLK3 has a more general role in the migration of other highly invasive breast cancer cell lines, a stable Mlk3-silenced population of MDA-MB-435 cells (Chambers, 2009), generated as described above, and Hs578T cells transiently transfected with an Mlk3 siRNA were subjected to transwell migration assays. In agreement with our findings in MDA-MB-231 cells, Mlk3 silencing decreased the migration of the MDA-MB-435 and Hs578T cells by two-fold (Figures 2c and d).

To assess whether the catalytic activity of MLK3 is required for breast cancer cell migration, a small ATP analog that selectively inhibits MLKs, CEP-11004 (Murakata *et al*., 2002; Shacka *et al*., 2006), was added

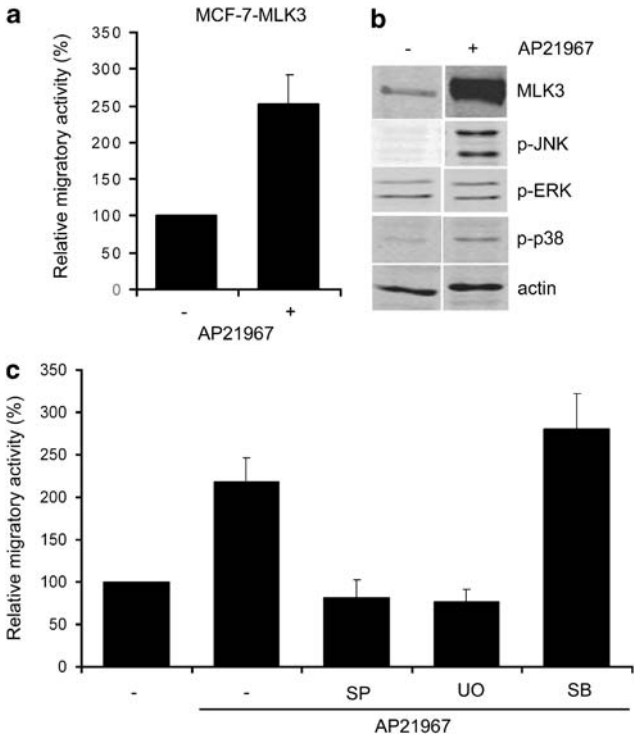


Figure 4 Induced expression of MLK3 promotes the migration of MCF-7 cells. (**a**) MCF-7-MLK3 cells were seeded in a Boyden chamber with a vehicle (–) or with 25 nM AP21967 and subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 10% FBS for 24 h. Triplicate wells were used for each of three independent experiments. The results are expressed relative to the migration of uninduced MCF-7-MLK3 cells (= 100%). Column: Mean of three experiments. Bar: s.e. *P*-value was determined by Student's *t*-test (**P* < 0.01). (**b**) MCF-7- MLK3 cells were treated with a vehicle (–) or 25 nM AP21967 for 20 h. Total cellular lysates containing 60 μg of total protein were analyzed by western blotting using the indicated antibodies. (**c**) MCF-7-MLK3 cells were seeded in a Boyden chamber in a medium containing the vehicle (–) or 25 nM AP21967, and the indicated inhibitors (SP = 15 μM SP600125, U0 = 10 μM U0126 or SB = 10 μM SB203580), and subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 10% FBS for 24 h. Triplicate wells were used for each of two independent experiments. The results are expressed relative to the migration of uninduced MCF-7-MLK3 cells (= 100%). Column: Mean of two independent experiments. Bar: s.e. FBS, fetal bovine serum; MLK3, mixed-lineage kinase-3.

to MDA-MB-231 cells in a transwell migration assay. As shown in Figure 3a, CEP-11004 inhibits the migration of the MDA-MB-231 cells by two-fold, supporting the notion that breast cancer cell migration depends on MLK activity. Consistent with the previously described catalytic roles of MLK3 in JNK and p38 signaling, CEP-11004 blocked the activation of JNK and p38, but not ERK, as judged by western blotting using phosphospecific antibodies (Figure 3b). CEP-11004 also dramatically inhibited closure in a wound-healing assay of MDA-MB-231 cells (Figure 3c). A JNK inhibitor, SP600125, decreased wound closure. As has been previously reported, the p38 inhibitor, SB203580, decreased the migration of MDA-MB-231 cells (Timoshenko *et al*., 2007). These data suggest that both p38 and JNK contribute to the migration of MDA-MB-231 cells, which may explain why the MLK inhibitor, which decreases both p38 and JNK activity, is more efficacious than either the JNK or p38 inhibitor in blocking the migration of these cells.

Induced expression of MLK3 promotes the migration of the poorly invasive MCF-7 breast cancer cells and of MCF10A mammary epithelial cells

In complementary experiments, we asked whether induced expression of active MLK3 could promote the migration of poorly invasive MCF-7 breast cancer cells (Zhang *et al*., 2004; Schachter *et al*., 2006) and MCF10A mammary epithelial cells. When a stable population of MCF-7–MLK3 cells was treated with the transcriptional inducer AP21967 and subjected to a transwell migration assay, a 2.5-fold increase in the migration of the MCF-7 cells (Figure 4a) was observed. Stable populations of MCF10A that inducibly express MLK3 were also generated and showed a two-fold increase in transwell migration upon transcriptional induction of MLK3 (Figure 5a). Induction of MLK3 potently induces JNK activation but has no effect on ERK activation in either MCF-7 or MCF10A cells. Induced expression of MLK3 results in a two-fold increase in p38 activity in both MCF-7 and MCF10A cells (Figures 4b and 5b). In addition, MLK3 induction had no effect on ERK-5 activation in MCF10A cells (Supplementary Figure 2).

To assess the contributions of the MAPK pathways to MLK3-induced MCF-7 and MCF10A cell migration, transwell migration assays were performed in the presence of MAPK pathway inhibitors. The JNK inhibitor SP600125 was effective in blocking the MLK3-induced migration of both MCF-7 and MCF10A cells (Figures 4c and 5c). The ERK pathway inhibitor also inhibited migration to a large extent, consistent with previous reports using these cell lines (Lester *et al*., 2005; Wilsbacher *et al*., 2006). Our findings in MCF-7 and MCF10A cells that U0126 blocks MLK3-induced migration, even though MLK3 does not enhance ERK activation, is consistent with the idea that basal ERK activity is required for migration of these cells. Notably, basal ERK activation is observed in the absence of MLK3 induction in both MCF-7 and MCF10A cells. The p38 inhibitor did not inhibit MLK3-induced migration in these cell lines, suggesting that MLK3-induced p38 activity is not sufficient to drive the MLK3-induced migration of MCF-7 or MCF10A cells.

Western blotting of cellular lysates, using phosphospecific antibodies against active JNK, ERK and p38, as well as MAPKAPK2, a downstream effector of p38 (Young *et al*., 1997), confirms that each inhibitor appropriately blocked its cognate pathway (Supplementary Figure 3). The increased levels of active JNK and active ERK observed (Supplementary Figure 3), upon treatment with the p38 inhibitor, likely reflect the previously reported antagonistic crosstalk between the p38 pathway and other MAPKs (Hall and Davis, 2002; Schachter *et al*., 2006), as this phenomenon was also observed in MDA-MB-231 cells (Supplementary Figure 4).

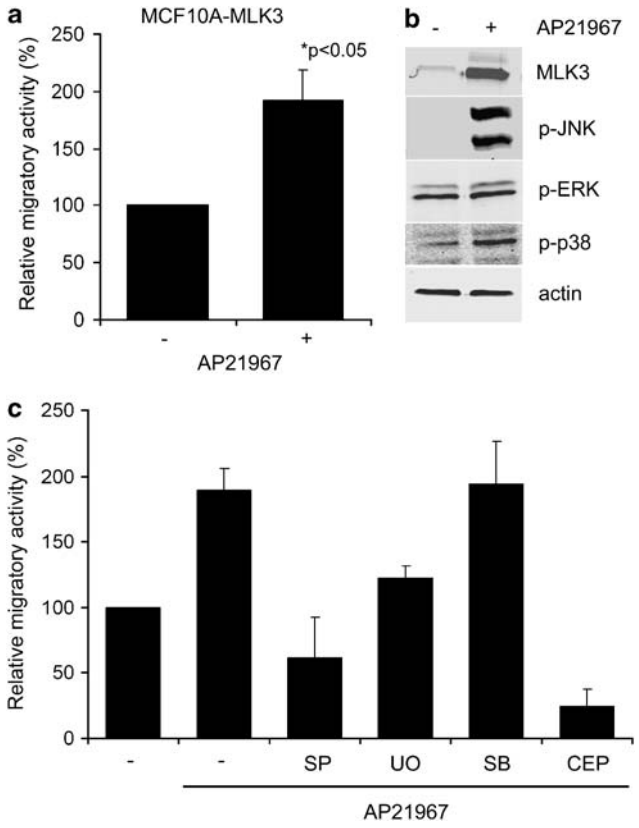


Figure 5 Induced expression of MLK3 promotes the migration of MCF10A cells. (**a**) MCF10A-MLK3 cells were seeded in a Boyden chamber with the vehicle or with 25 nM AP21967 and subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 2% horse serum for 24 h. Triplicate wells were used for each of three independent experiments. The results are expressed relative to the migration of uninduced MCF10A-MLK3 cells (=100%) (lower panel). Column: Mean of three experiments. Bar: s.e. *P*-value was determined by Student's *t*-test (*$P<0.05$). (**b**) MCF10A-MLK3 cells were treated with a vehicle (−) or with 25 nM AP21967 in media containing 2% horse serum and 20 ng/ml EGF for 20 h. Cellular lysates containing 60 μg of total protein were analyzed by western blotting using the indicated antibodies. (**c**) MCF10A-MLK3 cells were seeded in a Boyden chamber in a medium containing the vehicle (−) or 25 nM AP21967, and the indicated inhibitors (SP = 15 μM SP600125, U0 = 10 μM U0126, SB = 10 μM SB203580 or CEP = 400 nM CEP-11004), and subjected to a transwell migration assay as described under Materials and methods. The cells were allowed to migrate toward 2% horse serum for 24 h. Triplicate wells were used for each of two independent experiments. The results are expressed relative to the migration of uninduced MCF10A-MLK3 cells (=100%). Column: Mean of two independent experiments. Bar: s.e. EGF, epidermal growth factor; MLK3, mixed-lineage kinase-3.

Collectively, these data indicate that MLK3 expression is sufficient to activate JNK signaling, which is crucial for the MLK3-mediated migration of MCF-7 and MCF10A cells.

Induced expression of MLK3 increases mammary acinar size in an MLK3–JNK-dependent pathway

Three-dimensional cell culture systems provide a physiologically appropriate environment for assessing the effect of oncogenes on mammary epithelium (Debnath and Brugge, 2005; Lee *et al*., 2007). To determine whether MLK3 disrupts normal mammary morphogenesis, the inducible MLK3 expression system was used to express active MLK3 in preformed acinar structures composed of growth-arrested, polarized MCF10A cells. A population of stable, inducible MCF10A-MLK3 cells was seeded in Matrigel cultures under low epidermal growth factor (EGF) (0.1 ng/ml) and standard EGF (5 ng/ml) concentrations. Polarized, hollow acini were established by day 10 as judged by confocal sectioning of acini after 4′,6-diamidino-2-phenylindole and GM130 staining (data not shown). After 10 days, cultures were treated with AP21967 to induce the expression of MLK3. As is readily apparent at day 20 (Figure 6a), compared with uninduced MCF10A-MLK3 acini, induced expression of MLK3 dramatically increases acinar size under both EGF concentrations. Induction of MLK3 expression resulted in a 2.8- and a 2.0-fold increase in acinar cross-sectional area under low and standard EGF concentrations, respectively (Figure 6b). Data from a morphometric analysis of acinar cross-sectional area performed at day 20 are presented in a box-and-whiskers plot (Figure 6c). Using the corresponding control vector-expressing MCF10A cells, endogenous MLK3 levels (Supplementary Figure 5a) and acinar structures (Supplementary Figure 5b) were unaffected by AP21967. Highlighting the importance of the 3D environment for the MLK3-mediated effects on growth and morphogenesis, induced expression of MLK3 in MCF10A-MLK3 cells cultured on two-dimensional plastic does not induce proliferation (Supplementary Figure 1b).

Addition of CEP-11004 at the time of MLK3 induction prevents the increased size of MCF10A-MLK3 acini, indicating a requirement for MLK3 activity (Figure 6d). The JNK inhibitor also prevented the MLK3-induced phenotype. The inhibitors had no observable effects on uninduced mammary acini. These data indicate that active MLK3 signaling to JNK is required for the effects of induced MLK3 in 3D culture.

Induced MLK3 bypasses growth arrest, initiates luminal repopulation of preformed mammary acini and suppresses Bim expression

By day 20 in Matrigel culture, the acini derived from MCF10A cells should be growth-arrested (Debnath and Brugge, 2005). To determine whether induction of active MLK3 is sufficient to bypass growth arrest, acini were examined for expression of the proliferating-cell antigen, Ki-67, at day 20. As shown in Figure 7a, fewer than 10% of uninduced MCF10A-MLK3 acini contained Ki-67-positive cells in cultures containing both low and standard EGF concentrations, whereas greater than 70 and 90% of MLK3-induced acini contained at least one Ki-67-positive cell in cultures with low and standard EGF concentrations, respectively. It should be noted that although the majority of the MLK3-induced acini contained multiple proliferating cells, Ki-67-positive cells were still in the minority. AP21967 does not affect

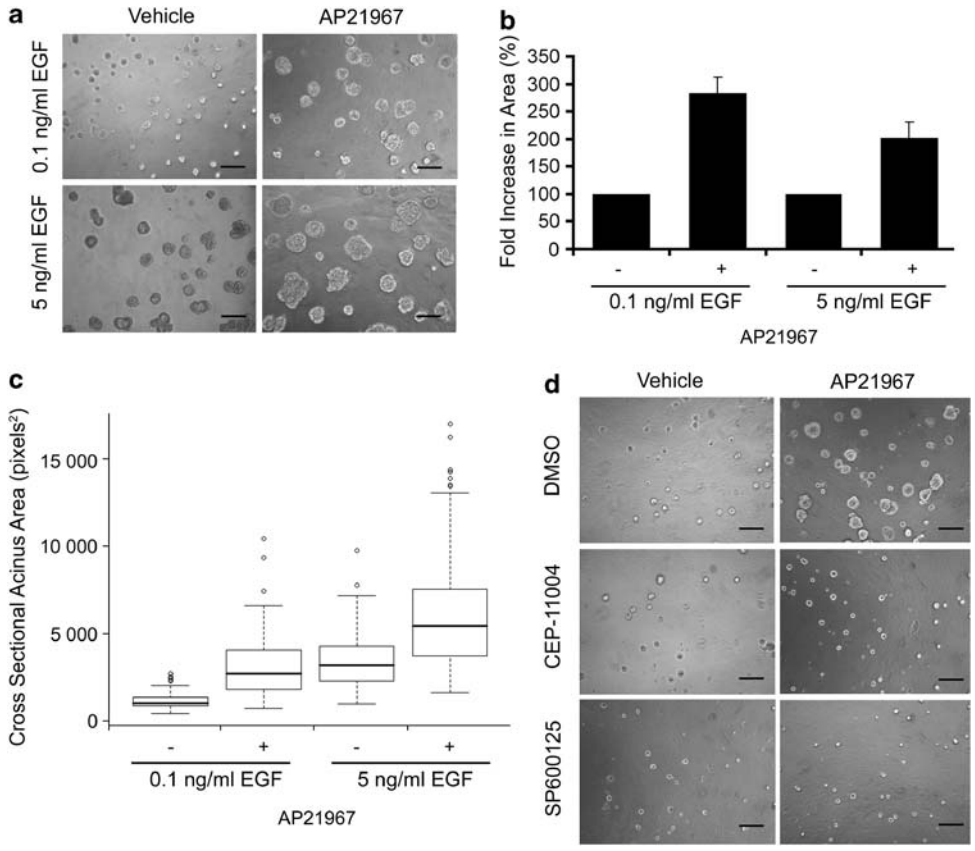


Figure 6 Induced expression of MLK3 increases the size of pre-established mammary acini. MCF10A-MLK3 cells were seeded in an overlay medium containing 0.1 or 5 ng/ml EGF on Matrigel as described under Materials and methods. (**a**) Fresh medium containing vehicle or 50 nM AP21967 was added to cultures on day 10 and images were acquired on day 20 of culture. Scale bar: 50 μm. (**b**) The cross-sectional area of MLK3-induced acini relative to that of uninduced acini (= 100%). Each column represents at least 300 acini per condition combined from three independent experiments. The error bar indicates the s.d. (**c**) Cross-sectional pixel areas of individual acini were determined as described under Materials and methods, and plotted as a box plot. Black line, median value; spread, interquartile range; circles, outliers. The data are from a representative set of three independent experiments where ~150 acini per condition were measured. (**d**) MCF10A-MLK3 cells were seeded in an overlay medium containing 0.1 ng/ml EGF on Matrigel as described under Materials and methods. Fresh medium containing a vehicle (−) or 50 nM AP21967, and the indicated inhibitor (400 nM CEP-11004 or 15 μM SP600125), was added on day 10 and images were acquired on day 18. Scale bar: 50 μm. Representative photographs from one of three independent experiments, each performed in duplicate wells. EGF, epidermal growth factor; MLK3, mixed-lineage kinase-3.

Ki-67 positivity in vector control cells (Supplementary Figure 5c). The ability of induced MLK3 to substantially increase mammary acinar size and proliferation under minimal EGF concentrations suggests that MLK3 signaling partially overrides the growth factor dependence of the mammary acini.

To determine whether increased acinar size caused by MLK3 affects polarity and luminal filling, structures were stained with nuclear and polarity markers, and optical sections through acini were examined. As shown in Figure 7b (left panel), at day 20, in the absence of AP21967, the MCF10A-MLK3 cells form uniform spherical structures with hollow lumens, whereas after culturing in the presence of AP21967 from day 10 to day 20, the lumens are largely filled, indicating that MLK3 can initiate luminal repopulation (Figure 7b, center and right panels). Similar luminal filling was observed upon MLK3 induction in cultures containing 0.1 ng/ml EGF (data not shown). Whereas induction of the oncogene ErbB2 promotes the formation of multi-acinar structures (Muthuswamy *et al*., 2001), the majority of the MLK3-expressing acini are still spherical (Figure 7b, central panel), with cell polarity largely maintained as determined by staining with the apical Golgi marker, GM130. However, some poorly defined mass-like structures with apparent disruption of cell polarity are also observed (Figure 7b, right panel). Taken together, these results show that expression of active MLK3 in mature acini reinitiates proliferation in growth-arrested structures, repopulates the lumen and can perturb cell polarity.

BimEL is a BH3-only proapoptotic protein that has been shown to be crucial in luminal apoptosis and lumen formation (Reginato *et al*., 2005). Oncogenes, like v-Src and ErbB2, which promote luminal filling of mammary acini, have been shown to suppress the expression of

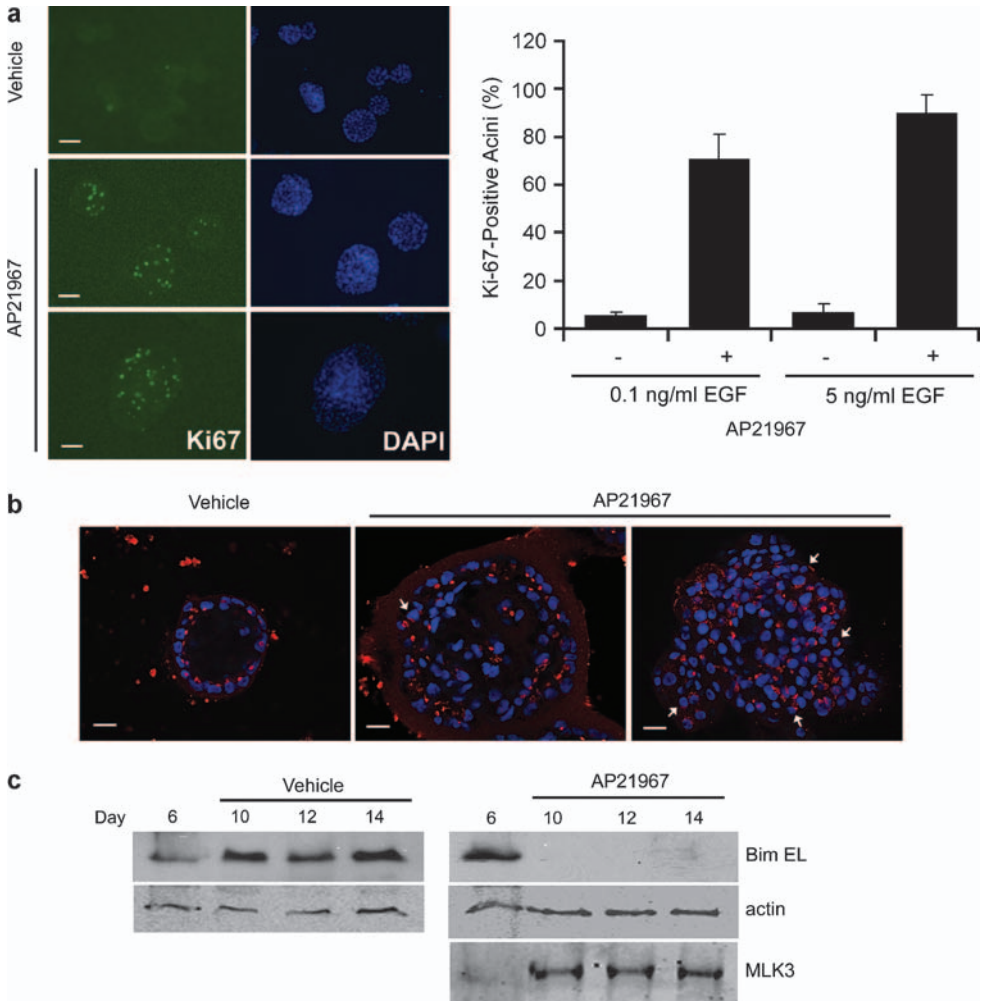


Figure 7 Induced expression of MLK3 in pre-established mammary acini promotes proliferation, luminal filling and disruption of polarity in growth-arrested structures. (**a**) MCF10A-MLK3 cells were seeded in an overlay medium containing 0.1 or 5 ng/ml EGF on Matrigel as described under Materials and methods. Fresh medium containing a vehicle (−) or 50 nM AP21967 was added to cultures on day 10. On day 20, the cultures were fixed and stained with anti-Ki-67 (green) and 4′,6-diamidino-2-phenylindole (blue). Representative images from cultures containing 5 ng/ml EGF. Scale bar: 50 μm (left panel). The cultures were scored for the number of acini containing one or more Ki-67-positive cells under the indicated conditions. Column: Percent Ki-67-positive acini based on at least 250 acini per condition combined from three independent experiments. Error bar: S.d. (right panel). (**b**) MCF10A-MLK3 cultures were established as above. On day 20, the cultures were fixed, stained with anti-GM130 (red) and 4′,6-diamidino-2-phenylindole (blue), and analyzed by confocal microscopy. Optical sections through the largest cross-sectional area of representative acini are shown. The white arrows indicate loss of polarity. Scale bar: 20 μm. (**c**) MCF10A-MLK3 cells were seeded in an overlay medium containing 5 ng/ml EGF on Matrigel as described under Materials and methods. Fresh medium containing a vehicle or 50 nM AP21967 was added to the cultures on day 7. On the indicated days in culture, cellular lysates were prepared as described under Materials and methods, and subjected to western blot analysis using the indicated antibodies. Actin was used as a loading control. The data are representative of two independent experiments. EGF, epidermal growth factor; MLK3, mixed-lineage kinase-3.

BimEL (Reginato *et al*., 2005). Consistent with these findings, upon MLK3 induction, we observe a dramatic decrease in BimEL levels on the basis of western blotting of lysates derived from 3D cultures of MCF10A-MLK3 cells (Figure 7c). These data show that, in the physiologically appropriate 3D context of mammary acini, MLK3 has both proliferative and antiapoptotic activities.

MLK3 induces the expression of proteins associated with invasive breast cancer

Our data indicate a central role for JNK in the MLK3-mediated malignant phenotypes. MLK3 can act as an MAP3K to activate JNK, which, in turn, can phosphorylate and activate c-jun, a component of the AP-1 transcription factor (Gallo and Johnson, 2002). We hypothesized that MLK3 signaling to JNK might activate c-jun to drive the expression of AP-1-regulated invasion genes in MCF10A cells. Notable AP-1-regulated invasion genes include c-jun (Karin, 1995; Minet *et al*., 2001; Zajchowski *et al*., 2001), Fra-1 (Bergers *et al*., 1995; Zajchowski *et al*., 2001) and vimentin (Rittling *et al*., 1989; Sommers *et al*., 1994b; Zajchowski *et al*., 2001; Rizki *et al*., 2007) Induced expression of MLK3 in MCF10A cells increases the level of c-jun (Figure 8a), Fra-1 (Figure 8b) and vimentin (Figure 8b),

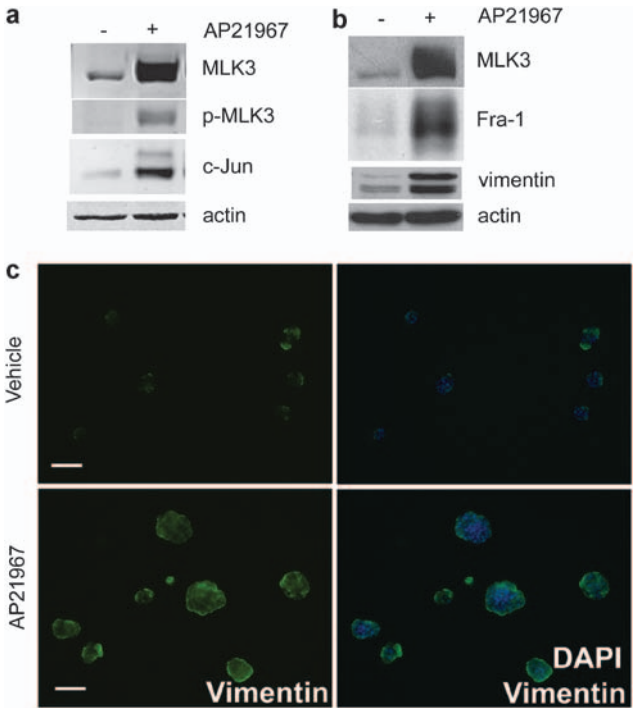


Figure 8 Induction of active MLK3 promotes the expression of the invasion-associated proteins c-jun, Fra-1 and vimentin. (**a**, **b**) MCF10A-MLK3 cells were seeded in growth media and induced with 25 nM AP21967 for 20 h. Cellular lysates containing 25 μg of total protein were subjected to western blotting using the indicated antibodies. The data are representative of three independent experiments. (**c**) MCF10A-MLK3 cells were seeded in 0.1 ng/ml EGF as described under Materials and methods. Expression of MLK3 was induced with 50 nM AP21967 on day 10, and on day 20 cultures were immunostained with anti-vimentin (green) and stained with 4′,6-diamidino-2-phenylindole (blue). The data are representative of three independent experiments. Scale bar: 50 μm. Fra-1, fos-related antigen-1; MLK3, mixed-lineage kinase-3.

supporting the notion that MLK3 promotes a malignant phenotype, at least in part, by controlling AP-1 activity. Notably, vimentin expression is also induced upon induction of MLK3 in preformed MCF10A mammary acini (Figure 8c).

MLK3 promotes invasion through signaling to AP-1

To investigate whether MLK3 regulates AP-1 activity to promote a malignant phenotype, the effect of MLK3 on the invasion of MCF10A cells was first examined. Induced expression of MLK3 in MCF10A-MLK3 cells increased invasion through Matrigel by three-fold in transwell invasion assays (Figure 9a). To test the requirement of AP-1 in MLK3-induced invasion, we made use of the dominant-negative mutant, Tam67 (Brown *et al*., 1993), which lacks the N-terminal trans-activating domain, as an inhibitor of AP-1 activity. Expression of pBabe-Tam67 (Johung *et al*., 2007), but not the pBabe control vector, effectively blocks MLK3-induced invasion (Figure 9a), indicating that MLK3 regulation of AP-1 activity is crucial for the invasive phenotype. As shown in Figure 9b, Tam67 is efficiently expressed in MCF10A-MLK3 cells and prevents the MLK3 induction of the AP-1-regulated invasion gene, Fra-1. In a complementary experiment, silencing of c-jun, an AP-1 component and a direct substrate of JNK, also blocks the MLK3-induced expression of Fra-1 (Figure 9c). These data, taken together, show that MLK3–JNK signaling increases AP-1 activity to promote the invasion of mammary epithelial cells.

Discussion

Identifying signaling pathways that contribute to breast cancer progression is principal to developing effective targeted therapies. MLK3 controls multiple MAPK signaling pathways and, depending on the cellular context, regulates proliferation, apoptosis, differentiation and migration. Several human breast cancer cell lines show higher levels of MLK3 compared with non-tumorigenic mammary epithelial cell lines (Figure 1). Using the Oncomine database (www.oncomine.com) (Rhodes *et al*., 2007), we found that MLK3 was among the top 10% overexpressed transcripts in DCIS as compared with normal breast tissue (Radvanyi *et al*., 2005), but it is also possible that MLK3 protein levels may be regulated at the translational and/or post-translational levels.

Using Mlk3 silencing (Figure 2) as well as an MLK-selective inhibitor (Figures 3 and 5) we provide evidence that MLK3 catalytic activity is crucial for migration of mammary cancer and epithelial cells. Studies with MAPK pathway inhibitors support the idea that MLK3–JNK signaling drives migration. Indeed, Slpr, the Mlk homolog in *Drosophila*, signals to JNK to promote epithelial sheet movement during the dorsal closure of the fly embryo (Stronach and Perrimon, 2002), in a genetically mapped pathway that leads to induction of JNK and AP-1 (Jacinto *et al*., 2002). Interestingly, using an MCF10A wound-healing assay, a large-scale RNA interference screen targeting over 1000 genes encoding all protein kinases and phosphatases, as well as a battery of proteins predicted to influence cell migration, identified over 60 genes as modulators of migration, one of which was *Mlk3*. (Simpson *et al*., 2008). A scaffolding function for MLK3 has been proposed for migration of A549 lung carcinoma cells (Swenson-Fields *et al*., 2008). However, our findings that an MLK inhibitor can block the migration of invasive breast cancer cells and that JNK inhibition blocks MLK3-induced migration in MCF-7 and MCF10A cells supports the idea that, at least in the context of breast cancer, MLK3–JNK signaling is crucial for cell migration. Indeed, a significant body of literature indicates a requirement for JNK in cancer progression (Cui *et al*., 2006; Ching *et al*., 2007; Dhillon *et al*., 2007; Khatlani *et al*., 2007; Vivanco *et al*., 2007; Su *et al*., 2009; Wagner and Nebreda, 2009).

It is interesting to note that both MDA-MB-231 and MCF-7 cells contain similar levels of MLK3, yet MDA-MB-231 cells are more migratory than MCF-7 cells. As MDA-MB-231 cells are highly malignant, it is likely

that multiple facets of the migratory machinery and signaling are aberrantly activated, contributing to their highly invasive phenotype. In MCF-7 cells, these signaling pathways are likely less active and thus high, induced MLK3–JNK signaling is needed to increase migration.

In DCIS, partial-to-complete filling of the luminal space is commonly observed and a poorer prognosis is associated with disruption of apicobasal polarity (Debnath and Brugge, 2005). 3D mammary epithelial cell cultures provide a structurally appropriate milieu for investigating breast cancer phenotypes. MCF10A cells grown in 3D Matrigel form growth-arrested, hollow spheroids composed of polarized cells that recapitulate many aspects of the normal mammary glandular architecture (Debnath and Brugge, 2005). Upon expression of certain oncogenes such as cyclin-D1 and HPV E7 (Debnath *et al*., 2002; Shaw *et al*., 2004; Debnath and Brugge, 2005), MCF10A acini grown in Matrigel increase in size but maintain apicobasal polarity and hollow lumens. A few oncogenes, such as ErbB2, Notch and the Ets family of transcription factors, are able to confer DCIS and invasive breast cancer traits on MCF10A cells, forming filled lumens and disorganized structures when grown in Matrigel cultures (Muthuswamy *et al*., 2001, Shaw *et al*., 2004). In this DCIS model, we induced the expression of active MLK3 in preformed, hollow MCF10A mammary acini and observed multiple features of DCIS, including bypass of growth arrest and re-initiation of luminal filling. The increase in the proliferative marker Ki-67 and suppression of the proapoptotic BimEL (Figure 7) upon MLK3 induction reveals both proliferative and novel antiapoptotic roles for MLK3 in the context of breast cancer.

The ability of MLK3 to regulate cell proliferation is highly dependent upon 3D culture in extracellular matrix. Growth of MDA-MB-231 cells in 3D Matrigel culture is largely stunted by silencing of MLK3 (data not shown), whereas proliferation on two-dimensional plastic is not enhanced by either induction of MLK3 in MCF10A cells or by silencing of Mlk3 in MDA-MB-231 cells (Supplementary Figure 1). These data indicate the importance of physiologically relevant 3D culture systems in assessing oncogenic phenotypes.

One of the major outcomes of MLK3–JNK signaling is phosphorylation of c-jun and activation of AP-1. In mammary epithelial cells, MLK3 induces the expression of c-jun, Fra-1 and vimentin whose genes are regulated by AP-1 and have been shown to be expressed at higher levels in invasive human breast cancer cell lines,

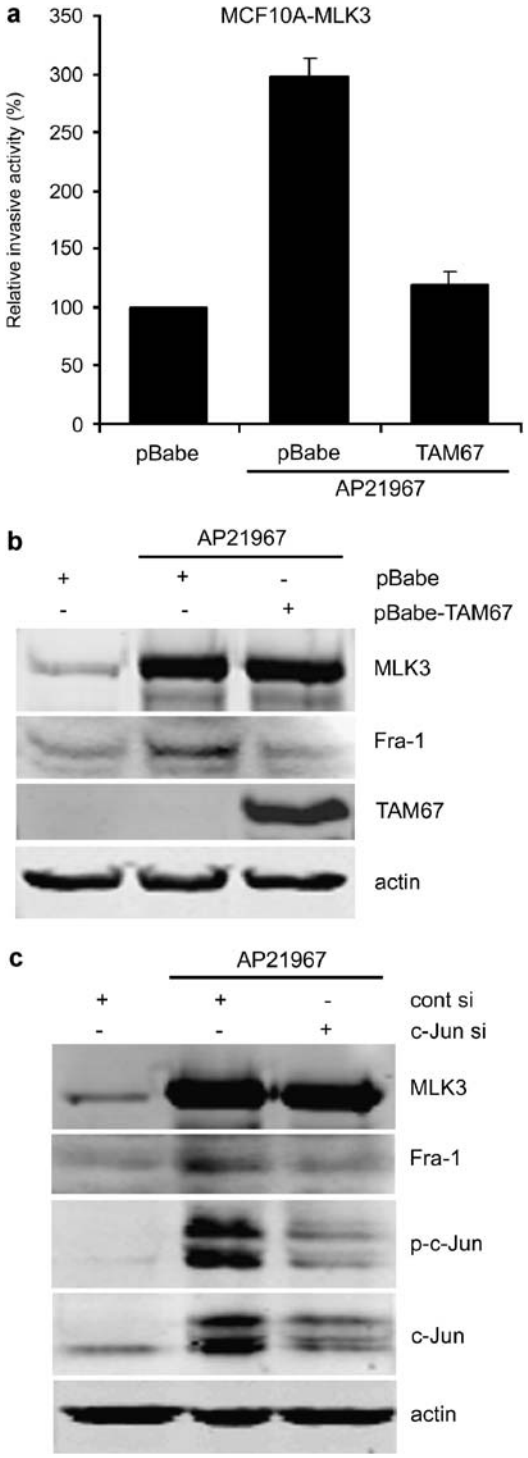


Figure 9 Induced expression of MLK3 promotes the invasion of MCF10A cells and MLK3-induced invasion requires AP-1 activity. (**a**) MCF10A-MLK3 cells carrying pBabe or pBabe-Tam67 retroviral vectors were seeded in a Matrigel-coated Boyden chamber with a vehicle or with 25 nM AP21967 and subjected to a transwell invasion assay as described under Materials and methods. The cells were allowed to migrate toward 2% horse serum for 24 h. Triplicate wells were used for each of two independent experiments. The results are expressed relative to the invasion of uninduced MCF10A-MLK3 cells (=100%). Column: Mean of two experiments. Bar: s.e. (**b**) MCF10A-MLK3 cells carrying pBabe or pBabe-Tam67 retroviral vectors were induced with 25 nM AP21967 for 20 h. Cellular lysates containing 25 μg of total protein were subjected to western blotting using the indicated antibodies. The data are representative of three independent experiments. (**c**) MCF10A-MLK3 cells were treated with a control or with c-jun siRNA for 24 h followed by addition of 25 nM AP21967 for 20 h. Cellular lysates containing 25 μg of total protein were subjected to western blotting using the indicated antibodies. The data are representative of three independent experiments. MLK3, mixed-lineage kinase-3.

compared with that in non-invasive breast cancer cell lines (Sommers *et al*., 1994a; Zajchowski *et al*., 2001). Vimentin is a classical marker for epithelial-to-mesenchymal transition, a transient cellular differentiation that allows cancer cell migration and invasion (Blick *et al*., 2008; Polyak and Weinberg, 2009). A subset of MLK3-expressing MCF10A acini also showed disruption of apicobasal polarity, a feature of invasive breast cancer. Furthermore, MLK3 promotes the invasion of MCF10A cells (Figure 9). Our finding that the dominant-negative AP-1 inhibitor, Tam 67, blocks MLK3-induced invasion indicates a crucial role for MLK3–JNK–AP-1 in invasion. Notably, decreased phospho-JNK1/2 levels are reported to correlate with improved overall survival in infiltrating ductal carcinoma (Yeh *et al*., 2006).

In summary, our investigation supports a crucial role for MLK3–JNK–AP-1 signaling in breast cancer cell migration and acquisition of a malignant phenotype. Although JNK has, in the past, been largely associated with stress signaling and neuronal apoptosis, a body of evidence linking JNK to cell migration, invasion and proliferation is accumulating. Our data are consistent with the idea that JNK signaling to AP-1 activates genes associated with epithelial-to-mesenchymal transition and a breast cancer invasive phenotype. Global gene expression analyses are underway to determine the repertoire of genes, including AP-1-regulated genes, affected by MLK3 in breast cancer. However, given the array of cellular substrates for JNK and other MLK3-regulated pathways, it will also be important to determine whether MLK3 signaling affects the cytoskeleton in the context of breast cancer. Finally, our finding that an MLK inhibitor is able to inhibit breast cancer cell migration and suppress the MLK3-induced phenotype of MCF10A cells in 3D culture indicates that MLK inhibitors may be useful in treating invasive breast cancer.

Materials and methods

Chemicals, antibodies, siRNAs and DNA constructs
For a complete list see theSupplementary information. Oligonucleotides for shRNAs targeting human MLK3 (Chadee and Kyriakis, 2004) were designed using OligoEngine 2.0, annealed and subcloned into the pSuper-retro vector (OligoEngine Inc., Seattle, WA, USA). Construction of the inducible vector encoding Flag-tagged MLK3, pLH-Z_{12}I-PL^2-MLK3, was previously described (Zhang *et al*., 2004).

Cell lines, cell culture, transfection and lysis
See Supplementary information for further details. Human mammary epithelial and breast cancer cell lines were from ATCC (Manassas, VA, USA). MCF-7 cells engineered to inducibly express MLK3 (MCF-7-MLK3) were previously described (Zhang *et al*., 2004). A population of MCF10A cells was engineered to inducibly express MLK3 (MCF10A-MLK3) or an empty vector control. Breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, PA, USA) with 10% fetal bovine serum (FBS). Transfection of siRNA (Mlk3-specific siRNA (10 nM), c-jun-specific siRNA (100 nM) or a universal control siRNA) was performed using InterferIN (Polyplus-transfection, New York, NY, USA) according to the manufacturer's instructions. Cells were lysed as described previously (Bock *et al*., 2000). The protein concentrations of the lysates were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). For detection of MLK3 protein levels in human mammary epithelial cells and breast cancer cells, the cells were harvested and lysed in radioimmunoprecipitation assay buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) containing protease inhibitors. The protein concentrations of the lysates were determined by the BCA protein assay (Pierce, Rockford, IL, USA). For detection of proteins in MCF10A cells grown in 3D culture, the cells were lysed by combining the Matrigel culture with an equal volume of radioimmunorecipitation assay lysis buffer containing protease inhibitors and passing through a 25-gauge needle; the lysates were clarified by centrifugation at 14 000 r.p.m. for 15 min before immunoblotting (Reginato *et al*., 2005).

Gel electrophoresis and western blot analysis
Cellular lysates were resolved by SDS–polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes and immunoblotted using appropriate antibodies followed by treatment with a horseradish peroxidase-conjugated or an IRDye-conjugated secondary antibody and developed by the chemiluminescence method or visualized by fluorescence (Li-COR Biosciences, Lincoln, NE, USA), respectively.

Transwell migration and invasion assays
Chemotactic migration or invasion was quantified using a Boyden chamber transwell assay (8 μm pore size; Corning Costar, Cambridge, MA, USA) using uncoated or Matrigel-coated filters, respectively. Cells were deprived of serum overnight, trypsinized and introduced into the upper chamber (5×10^4 for MDA-MB-231, MDA-MB-435 and Hs578T (6 h); 10^5 for MCF-7-MLK3 and MCF10A-MLK3 (24 h)). For the 24-h assays, mitomycin-C was included. The chemoattractant in the lower chamber was medium supplemented with 5% FBS (MDA-MB-231, MDA-MB-435 and Hs578T), 10% FBS (MCF-7-MLK3) or 2% horse serum (MCF10A-MLK3). The cells were fixed and stained as described by Goicoechea *et al*. (2009). Migrated cells in five randomly chosen fields were counted. The experiments were performed in triplicate wells and each experiment was performed at least two or three times as indicated.

Wounding-healing assay
MDA-MB-231 cells and derivatives were grown to confluence. The growth medium was replaced with fresh medium supplemented with mitomycin-C (1 μg/ml) and the monolayer of cells was scratched using a 200 μl pipette tip. Wound width was monitored over time by microscopy. Percentage wound recovery was expressed as follows: [1–(Width of the wound at a given time/width of the wound at $t = 0$)] × 100%.

3D morphogenesis assay
See Supplementary information for details. A single-cell suspension of 5000 cells was seeded per well on solidified Matrigel (BD Biosciences, San Jose, CA, USA) in overlay media (Debnath *et al*., 2003; Lee *et al*., 2007). After formation of mature acini, at day 10, MLK3 expression was induced with 50 nM AP21967 and cultures were assessed on day 20. All immunofluorescence procedures were performed as previously

described (Debnath *et al*., 2003). Acinar structures at day 20 were analyzed for size distribution using Metamorph.

Conflict of interest

The authors declare no conflict of interest.

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Cancer Research

MLK3 Regulates Paxillin Phosphorylation in Chemokine-Mediated Breast Cancer Cell Migration and Invasion to Drive Metastasis

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MLK3 Regulates Paxillin Phosphorylation in Chemokine-Mediated Breast Cancer Cell Migration and Invasion to Drive Metastasis

Jian Chen[1] and Kathleen A. Gallo[2,3]

Abstract

MLK3 kinase activates multiple mitogen-activated protein kinases and plays a critical role in cancer cell migration and invasion. In the tumor microenvironment, prometastatic factors drive breast cancer invasion and metastasis, but their associated signaling pathways are not well-known. Here, we provide evidence that MLK3 is required for chemokine (CXCL12)-induced invasion of basal breast cancer cells. We found that MLK3 induced robust phosphorylation of the focal adhesion scaffold paxillin on Ser 178 and Tyr 118, which was blocked by silencing or inhibition of MLK3-JNK. Silencing or inhibition of MLK3, inhibition of JNK, or expression of paxillin S178A all led to enhanced Rho activity, indicating that the MLK3–JNK–paxillin axis limits Rho activity to promote focal adhesion turnover and migration. Consistent with this, *MLK3* silencing increased focal adhesions and stress fibers in breast cancer cells. *MLK3* silencing also decreased the formation of breast cancer lung metastases *in vivo*, and breast cancer cells derived from mouse lung metastases showed enhanced Ser 178 paxillin phosphorylation. Taken together, our findings suggest that the MLK3–JNK–paxillin signaling axis may represent a potential therapeutic target and/or prognostic marker in breast cancer metastasis. *Cancer Res; 72(16); 4130–40. ©2012 AACR.*

Introduction

Recent decreases in breast cancer mortality are primarily because of improved diagnosis and treatment. However, approximately 40,000 deaths annually in the United States are due to breast cancer (1), primarily from metastasis to distant organs. Metastasis is a multistep process requiring tumor cell migration, intravasation, survival in circulation, extravasation, and colonization to a secondary site. Interrupting the metastatic process is key to reducing breast cancer mortality.

Chemokines and growth factors drive breast cancer migration, invasion, and metastasis. The chemokine, CXCL12/SDF1-α, binds its G-protein–coupled receptor, CXCR4, to promote cytoskeletal remodeling and migration in human breast cancer cells (2), and CXCL12-CXCR4 signaling is critical for breast cancer metastasis in mouse xenograft models (2, 3). High levels of CXCR4 are found in breast tumor cells isolated from pleural effusions (4) and correlate with lymph node metastases (5) and poor overall survival in patients (5, 6). Hepatocyte growth factor/scatter factor (HGF), through binding to its receptor, c-met, promotes cell motility and invasion (7). Aberrant c-met signaling and the *MET* oncogene are associated with basal breast cancer (8), and c-met overexpression predicts poor outcome in breast cancer patients (9, 10).

Mitogen-activated protein kinase (MAPK) signaling contributes to breast cancer cell migration, invasion, and metastasis. MLK3 is a MAPKKK that regulates the 3 major MAPK pathways (11). MLK3 contains an N-terminal SH3 domain, followed sequentially by a serine/threonine kinase domain, leucine zippers, a Cdc42/Rac interactive binding (CRIB) motif, and a C-terminal proline-rich region. MLK3 is autoinhibited through its SH3 domain. Binding of GTP-bound Rac or Cdc42 through MLK3's CRIB motif disrupts SH3-mediated autoinhibition and promotes zipper-mediated homodimerization, resulting in transautophosphorylation within the kinase domain, yielding the active kinase (12–14). MLK3 regulates cancer cell migration and invasion (15–19). We recently showed that MLK3 signaling through JNK to the transcription factor AP-1 is required for migration and invasion in human mammary basal epithelial and breast cancer cells. Activation of the MLK3-JNK-AP1 signaling axis induces expression of several breast cancer invasion genes (16).

The mechanics of cancer cell migration involve cytoskeletal remodeling and focal adhesion dynamics (20). Paxillin is a multidomain adaptor protein that localizes to focal adhesions, the multiprotein complexes that bridge the extracellular matrix and cytoskeleton. The dynamics of focal adhesion assembly and disassembly are controlled by protein interactions and phosphorylation events within the paxillin signaling hub. Paxillin, itself, is phosphorylated at numerous sites (21). Recruitment of FAK to focal adhesions and subsequent

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tyrosine phosphorylation of paxillin leads to focal adhesion disassembly (21–23). Phosphorylation of paxillin on Ser 178 by JNK is required for focal adhesion disassembly and migration in a basal breast cancer cell line (24).

Small Rho family GTPases include Rac, Cdc42, and Rho (25). Temporal and spatial activation of Rho GTPases is tightly controlled in migrating cells (20). Disrupting the activity cycle of Rho family GTPases results in inefficient cell migration (26). Cdc42 regulates formation of filopodia, whereas Rac controls formation of lamellipodia. Rho triggers formation of stress fibers and regulates focal adhesions (25). Phosphorylation of paxillin on Tyr 31 and Tyr 118 leads to enhanced Rac activity and decreased RhoA activity (27, 28).

Herein, we show that MLK3 is required for migration and invasion in response to CXCL12, and both CXCL12 and HGF signal to JNK through MLK3. We provide evidence that MLK3-activated JNK phosphorylates paxillin on Ser178 that, in turn, recruits FAK to paxillin, resulting in tyrosine phosphorylation. Disruption of the MLK3–JNK–paxillin signaling pathway increases Rho activity, focal adhesions, and stress fibers in basal breast cancer cells. *MLK3* silencing decreases formation of breast cancer lung metastases in a mouse xenograft model. In addition, breast cancer cells derived from mouse lung metastases show enhanced Ser 178 paxillin phosphorylation, which can be blocked by an MLK inhibitor. On the basis of these findings, we propose that targeting the MLK3–JNK–paxillin signaling axis may be a useful strategy to combat breast cancer metastasis.

Materials and Methods

Cell lines, chemicals, antibodies, DNA constructs, and siRNAs

Human mammary epithelial and breast cancer cell lines were from American Type Culture Collection. MDA-MB-231-luc2-tdTomato cells were from Caliper Life Sciences. Cell line authentication was carried out using short tandem repeat and amelogenin profiling. The p-paxillin (S178) antibody was from Bethyl Laboratories. All other phosphoantibodies were from Cell Signaling Biotechnology. Other antibody suppliers were Santa Cruz Biotechnology (ERK, JNK, and FAK), Sigma (Anti-Flag M2, HA, vinculin and actin), Millipore (paxillin), Abcam (CD44), and Clontech (GFP). MLK3 antibody was homemade or from Epitomics. Recombinant human CXCL12 was from R&D systems. Collagen I and Matrigel were from Becton Dickinson. Pharmacologic inhibitors SP600125, U0126, and SB203580 were from Calbiochem. CEP-1347 was kindly provided by Cephalon, Inc., a wholly-owned, indirect subsidiary of Teva Pharmaceutical Industries Ltd. Flag-MLK3 or MLK3 K144R constructs were described (14). GFP-FAK was a gift from Dr. Jun-Lin Guan (University of Michigan, Ann Arbor, MI). HA–Pax_{S178A} construct was generated from wild-type HA-Pax construct (a gift from Dr. Ravi Salgia, University of Chicago, Chicago, IL) using site-directed mutagenesis (Stratagene) following manufacturer's instructions. #1 *MLK3* siRNA (5′-GGGCAGUGACGUCUGGAGUUU-3′) and #2 *MLK3* siRNA (5′-CUGGAGGACUCAAGCAAUG-3′) were from Dharmacon (11, 15). JNK1/2 siRNA (5′-AAAGAAUGUCCUACCUUCU-3′) was from Qiagen (29). AP21967 was provided by Ariad Pharmaceuticals.

Stable cell populations and transfections

MCF10A-MLK3 cells (16) were treated with ± 50 nmol/L AP21967 to induce MLK3 expression. MDA-MB-231 cells expressing pSuper or *MLK3* short hairpin RNA (shRNA) have been described (16). Transfection of DNA constructs was carried out using Lipofectamine 2000 (Invitrogen). Transfection of siRNA (30–100 nmol/L) was carried out using INTERFERin (Polyplus transfection). Forty-eight hours posttransfection, cells were subjected to migration, invasion assays, immunofluorescence, or immunoblotting.

Immunoblotting, coimmunoprecipitations, and Rho GTPase assays

Preparation of cellular lysates and immunoblotting was as previously described (16). Western blots were developed by chemiluminescence or by fluorescence using LI-COR Odyssey infrared imaging (LI-COR). Coimmunoprecipitation experiments were carried out as described (13). Rho-GTP was measured using the Rhotekin-RBD pulldown assay (Cytoskeleton). Briefly, cells were lysed in ice-cold Triton X-100 lysis buffer and cleared cellular extracts (500 μg) were incubated with Rhotekin-RBD agarose beads (10 μg). Beads were pelleted, washed, and resuspended in 1.5× SDS sample buffer. GTP-bound Rho was detected by immunoblotting.

Migration and invasion assays

Chemotactic migration was quantified using a Boyden chamber transwell assay as described (16). The chemoattractant was 100 ng/mL CXCL12. For invasion assays, chambers were coated with Matrigel (1:5 dilution in DMEM/F12).

Immunofluorescence

Formaldehyde-fixed cells were permeabilized with 0.5% Triton X-100, blocked in 4% bovine serum albumin, and stained with anti-vinculin antibody (1:200 dilution), followed by Alexa Fluor-488–conjugated anti-mouse IgG (1:200 dilution). To visualize stress fibers, cells were stained with Alexa Fluor-546–conjugated phalloidin (1:50, Invitrogen), and nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI, 0.5 μg/mL). Images were acquired using an Olympus FV1000 confocal laser scanning microscope. Focal adhesions were quantified using ImageJ software.

Spontaneous metastasis model

All experiments involving animals were carried out in accordance with standard protocols approved by All University Committee on Animal Use and Care at Michigan State University. Female athymic nu/nu mice (6-week-old; Harlan Laboratory) were maintained in microisolation cages under specific pathogen-free conditions. MDA-MB-231 cells-pSuper or -sh*MLK3* (2×10^6 cells/site) were resuspended in PBS and Matrigel (1:1 v/v) and surgically inoculated bilaterally into No. 4 mammary glands. Tumors were measured using a caliper twice weekly. Mice were euthanized after 7 weeks, primary tumors were excised and lysed using radioimmunoprecipitation

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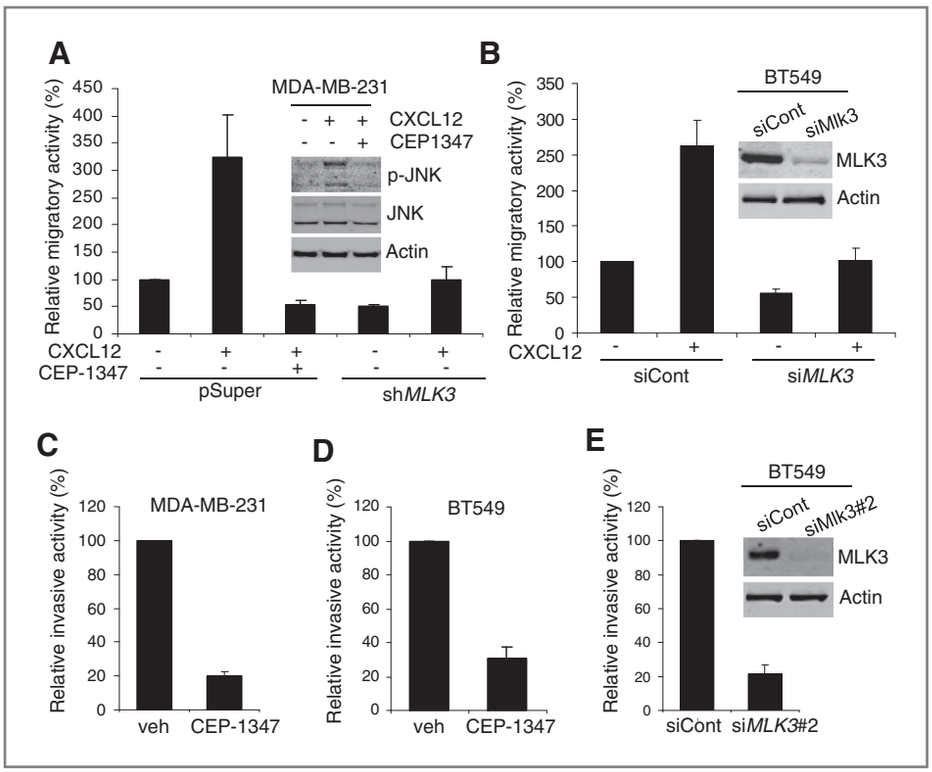

Figure 1. Silencing or inhibition of MLK3 blocks CXCL12-induced migration and invasion. A, serum-deprived MDA-MB-231-pSuper or MDA-MB-231-pSuper-sh*MLK3* were treated with CXCL12 (100 ng/mL) ± CEP-1347 (400 nmol/L) for 30 minutes. Total cellular lysates were analyzed by immunoblotting using indicated antibodies (top). MDA-MB-231-pSuper or MDA-MB-231-pSuper-sh*MLK3* were allowed to migrate toward CXCL12 (100 ng/mL) for 24 hours. Migrated cells were quantified as described (16). Column, mean of 3 experiments. Bar, SE. B, BT549 cells were treated with control or *MLK3* siRNA for 48 hours, serum deprived, and allowed to migrate toward CXCL12 (100 ng/mL) for 48 hours. C, MDA-MB-231 cells, pretreated ± 400 nmol/L CEP-1347 for 6 hours in serum-free medium, were allowed to invade toward CXCL12 (100 ng/mL) for 24 hours. D, BT549 cells were pretreated by ± 400 nmol/L CEP-1347 and CXCL12-induced transwell invasion was determined as in B. E, BT549 cells were treated as in B and subjected to a transwell invasion assay. Column, mean of 4 experiments. Bar, SE.

assay buffer. Mouse lungs were fixed in formalin overnight and paraffin sections were analyzed using anti-CD44–specific human antibody and Vectastain Elite ABC kits and DAB Substrate (Vector laboratory). Metastatic nodules were quantified in 10 lung sections per mouse, and statistical analysis was carried out using GraphPad Prism 5.

Experimental metastasis model

MDA-MB-231-Luc2-tdTomato cells (10^6 in 100 μL saline) were injected into tail vein of nude mice. Weekly, following intraperitoneal injection with D-luciferin (150 mg/kg), mice were imaged using the Caliper IVIS Spectrum. After 12 weeks, lungs containing MDA-MB-231 metastases were extracted, minced, and cultured in puromycin (2 μg/mL). Recovered MDA-MB-231 cells were designated as MDA-MB-231 Lu cells.

Results

MLK3 is required for migration and invasion of basal breast cancer cells toward CXCL12

CXCL12 is critical in breast cancer cell migration and metastasis (3, 30). The highly invasive, basal-like breast cancer cell lines, MDA-MB-231 and BT549, both express high levels of CXCR4 (30). In a transwell migration assay, CXCL12 increased migration of MDA-MB-231 cells expressing control vector by approximately 3-fold. MLK3 expression is efficiently ablated in a stable population of MDA-MB-231 cells (16). CXCL12-induced migration was completely blocked in MDA-MB-231 cells stably expressing shMlk3 or treated with CEP-1347, a selective MLK inhibitor (Fig. 1A). As shown in Fig. 1A, CXCL12 activated JNK. To confirm the efficacy of CEP-1347, we used phospho-JNK (p-JNK) as a readout for active MLK signaling. Immunoblotting using a p-JNK antibody showed that CEP-1347 blocked CXCL12-induced JNK activation (Fig. 1A). In addition, transient silencing of *MLK3* in BT549 cells reduced CXCL12-induced migration (Fig. 1B).

In a Matrigel invasion assay using CXCL12 as a chemoattractant, CEP-1347 reduced invasion of MDA-MB-231 cells by approximately 5-fold (Fig. 1C). In addition, CEP-1347 blocked invasion of BT549 cells (Fig. 1D). Silencing of *MLK3* in BT549 cells also largely inhibited invasion (Fig. 1E). *MLK3* silencing had negligible effect on proliferation of BT549 cells (Supplementary Fig. S1). Taken together, these results supported the idea that MLK3 signaling is required for migration and invasion of invasive basal breast cancer cells in response to CXCL12.

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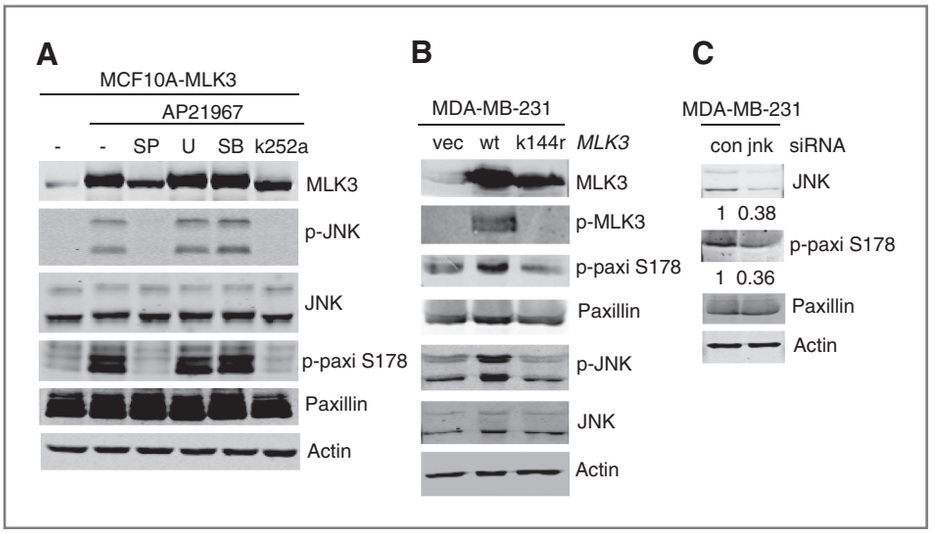

Figure 2. MLK3-JNK signaling promotes phosphorylation of paxillin at Ser 178. A, MCF10A-MLK3 cells were treated ± 50 nmol/L AP21967 for 20 hours with indicated inhibitors: 15 μmol/L SP600125, 10 μmol/L U0126, 10 μmol/L SB203580, or 400 nmol/L K252a for an additional 24 hours. Cellular lysates were analyzed by Western blotting. B, MDA-MB-231 cells were transfected by wild-type or MLK3 K144R vector for 24 hours. Cellular lysates were analyzed by Western blotting. C, MDA-MB-231 cells were treated with control or JNK1,2 siRNA for 48 hours. Cellular lysates were analyzed by immunoblotting. Quantitation of Western blots normalized to actin was carried out using LI-COR Odyssey software V3.0.

Active MLK3 promotes phosphorylation of paxillin through JNK

We recently showed that induced expression of MLK3 promotes migration and invasion of MCF10A mammary epithelial cells, which requires JNK-AP1 (16). We hypothesized that, in addition to its impact on gene expression, MLK3-JNK signaling might act upon cytoskeleton or focal adhesions to regulate cell migration and invasion. Phosphorylation of Ser 178 of paxillin by JNK is necessary for focal adhesion turnover and cell migration (24).

To investigate whether MLK3 can promote phosphorylation of paxillin, MCF10A cells engineered to inducibly express MLK3 were used (16). Upon MLK3 induction, JNK was activated and robust Ser 178 phosphorylation of paxillin was observed. Inhibition of MLKs with K252a, or of JNK with SP600125, blocked phosphorylation of paxillin at Ser 178. In contrast, inhibition of ERK signaling with U0126, or p38 with SB203580, had no effect on MLK3-induced paxillin phosphorylation (Fig. 2A). Like K252a, its derivative CEP-1347 blocked MLK3-induced paxillin phosphorylation (Supplementary Fig. S2). In MDA-MB-231 cells, transient expression of wild-type MLK3, but not kinase inactive, MLK3 K144R, induced JNK activation and Ser 178 phosphorylation of paxillin (Fig. 2B). Furthermore, silencing of JNK1/2 in MDA-MB-231 cells decreased Ser 178 phosphorylation of paxillin (Fig. 2C), confirming the requirement for JNK in paxillin phosphorylation. Thus the MLK3-JNK signaling axis promotes paxillin phosphorylation at Ser 178.

Chemokine and growth factor induce paxillin phosphorylation in an MLK3-dependent manner

To determine whether MLK3 is required for phosphorylation of paxillin at Ser 178, serum-deprived MDA-MB-231 cells stably expressing control or sh*MLK3* vector were treated with 10% serum and paxillin phosphorylation was assessed. Serum treatment led to JNK activation and maximal phosphorylation of paxillin at Ser 178 at 30 minutes, both of which were largely abrogated in cells expressing sh*MLK3* (Fig. 3A), suggesting MLK3 is a major mediator of JNK signaling to paxillin. As phosphorylation of paxillin on Ser 178 is associated with breast cancer migration, we investigated the impact of CXCL12 and HGF on paxillin phosphorylation. Both factors induced JNK activation and phosphorylation of paxillin at Ser 178 in MDA-MB-231 cells, which was reduced by CEP-1347 (Fig. 3B).

Likewise, in BT549 cells, based on 4 independent experiments, both CXCL12 and HGF induced JNK activation (3- and 3.4-fold, respectively) and paxillin phosphorylation at Ser 178 (3.2- and 4.4-fold, respectively), which was attenuated by silencing with *MLK3* siRNA (Fig. 3C). MLK inhibition with CEP-1347 also reduced both JNK activation and Ser 178 paxillin phosphorylation (Fig. 3D). The requirement of MLK3 in paxillin phosphorylation was confirmed using a different siRNA sequence (Supplementary Fig. S3A and B). In BT549 cells, HGF potently activated ERK, whereas only a small increase in ERK activation was observed in response to CXCL12. ERK activation was refractory to the MLK inhibitor, consistent with the proposed scaffolding role of MLK3 in ERK activation (11). Because MDA-MB-231 cells harbor activating mutations in both Ras and Raf (31), resulting in constitutive ERK activation, it is not too surprising that CXCL12 and HGF have relatively little effect on ERK activity in these cells. These data showed a requirement for active MLK3 in JNK activation and Ser 178 paxillin phosphorylation, triggered by either a prometastatic chemokine or growth factor in basal breast cancer cells.

MLK3 controls Tyr 118 phosphorylation of paxillin and its association with focal adhesion kinase

Cell migration requires efficient assembly and disassembly of focal adhesion complexes. Paxillin undergoes phosphorylation

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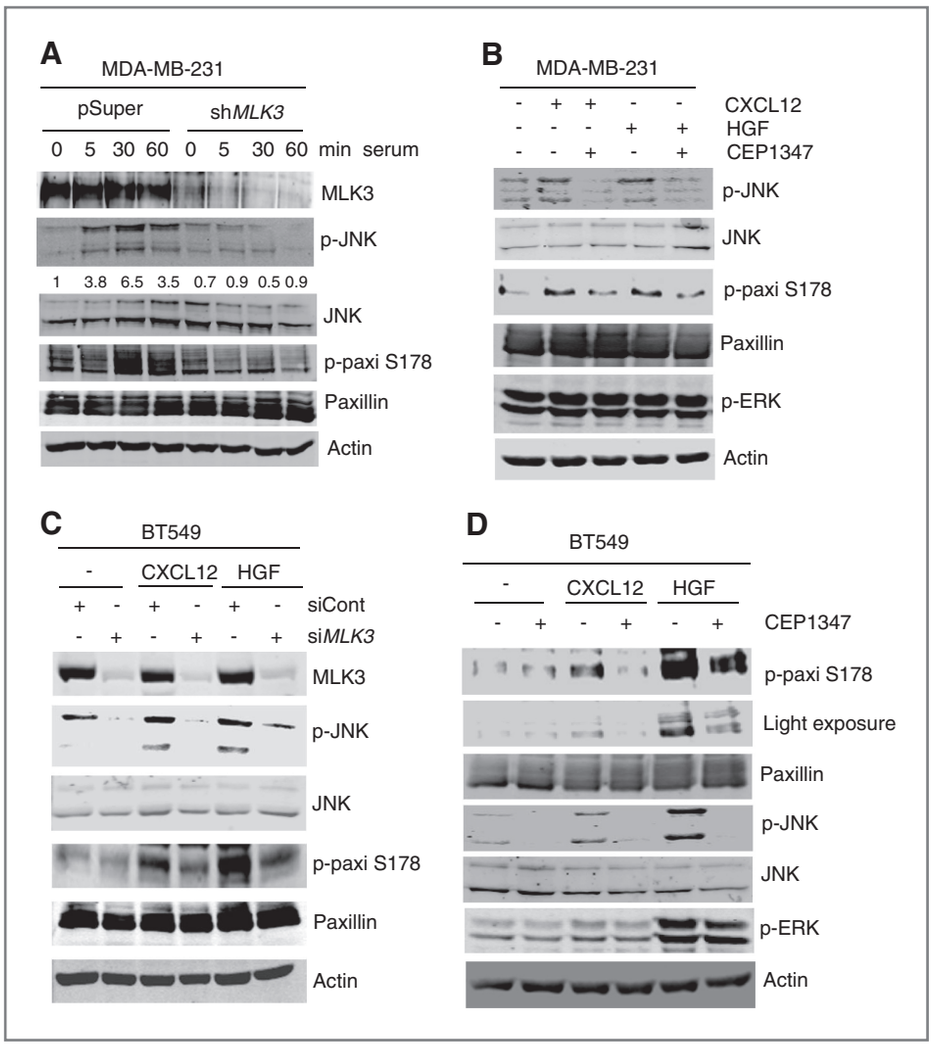

Figure 3. *MLK3* silencing or an MLK inhibitor impairs paxillin phosphorylation at Ser 178. A, serum-deprived MDA-MB-231-pSuper or pSuper-sh*MLK3* were treated with 10% serum for indicated times. Total cellular lysates were analyzed by immunoblotting. Quantitation of p-JNK/JNK determined by LI-COR Odyssey software V3.0 is shown. B, serum-deprived MDA-MB-231 cells were treated with ± CEP-1347 (400 nmol/L) for 6 hours, followed by CXCL12 (100 ng/mL) or HGF (100 ng/mL) for 30 minutes. C, BT549 cells were transfected with control or *MLK3* siRNA and treated for 30 minutes with 100 ng/mL CXCL12 or HGF. D, serum-deprived BT549 cells were treated with ± CEP-1347 (400 nmol/L) for 6 hours, followed by 100 ng/mL CXCL12 or HGF for 30 minutes.

at multiple sites to modulate protein–protein interactions in focal adhesions (21). The role of Ser 178 phosphorylation of paxillin is not completely clear. In corneal epithelial cells, Ser 178 phosphorylation of paxillin recruits FAK to mediate tyrosine phosphorylation of paxillin (32).

As MLK3 controls Ser 178 phosphorylation of paxillin, we tested whether MLK3 indirectly modulates tyrosine phosphorylation of paxillin and regulates interactions among focal adhesion proteins. Ectopic expression of MLK3 in BT549 cells promoted both Ser 178 and Tyr 118 paxillin phosphorylation, showing that active MLK3 drives Tyr 118 phosphorylation of paxillin (Fig. 4A). In response to serum stimulation in MDA-MB-231 cells, CEP-1347 reduced Tyr 118 phosphorylation of paxillin by 2-fold at 5 minutes and 3.5-fold at 20 minutes, paralleling the effects of JNK inhibition (Fig. 4B). Consistently, HGF-induced Tyr 118 phosphorylation of paxillin was dramatically reduced in MDA-MB-231 cells expressing sh*MLK3* (Fig. 4C). From these data, we concluded that MLK3 is critical for Tyr 118 phosphorylation of paxillin.

To test whether MLK3 influences association of paxillin with FAK, we took advantage of 293T cells as an efficient cotransfection system. Ectopically expressed GFP-FAK and HA-paxillin showed weak association in coimmunoprecipitations from serum-deprived 293T cells (Fig. 4D). EGF has previously been shown to facilitate the interaction between FAK and paxillin in 293T cells (32). Our data confirmed that the association between GFP-FAK and HA-paxillin is enhanced by EGF (Fig. 4D). However, pretreatment with CEP-1347 abrogated the EGF-induced association of GFP-FAK and HA-paxillin (Fig. 4D). We were able to detect endogenous paxillin in a FAK immunoprecipitate from MDA-MB-231 cells in growth medium, which was reduced by CEP-1347. In the immunoprecipitated FAK complex, levels of Ser 178 phosphorylated paxillin and total paxillin correlate directly, consistent with the idea that Ser 178 phosphorylation drives association of FAK with paxillin (Fig. 4E). Conversely, forced expression of active Flag-MLK3 in MDA-MB-231 cells increased interaction of endogenous paxillin and FAK as well as phosphorylation of paxillin at both Ser 178 and Tyr 118 (Fig. 4F). These data provided strong evidence that MLK3 regulates both paxillin phosphorylation and FAK-paxillin interactions.

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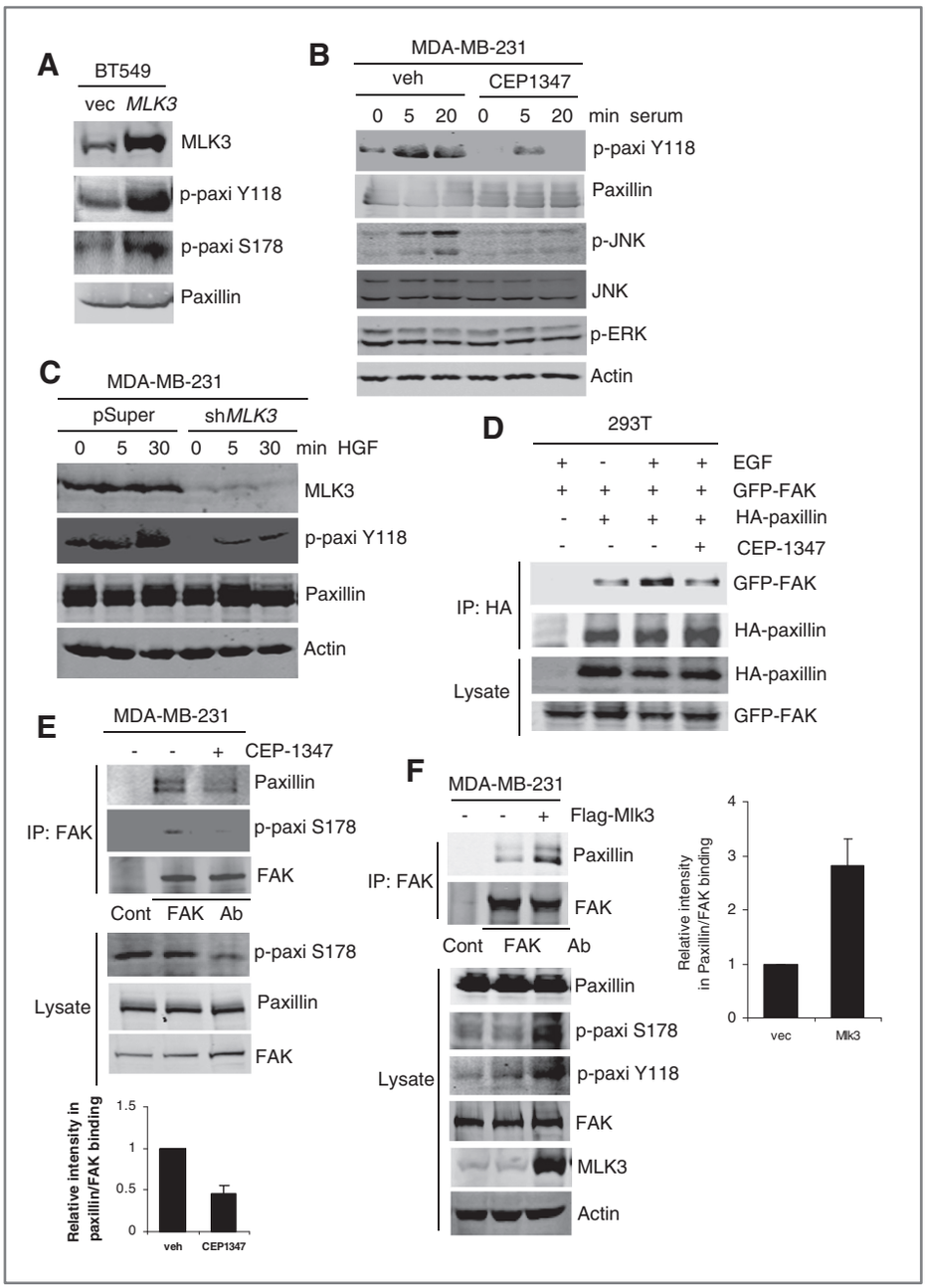

Figure 4. MLK3 promotes Tyr 118 phosphorylation of paxillin and interaction of FAK with paxillin. A, immunoblots of cellular lysates from BT549 cells transfected with control or Flag-*MLK3* vector are shown. B, serum-deprived MDA-MB-231 cells were treated ± 400 nmol/L CEP-1347 for 6 hours followed by 10% serum for indicated times. Immunoblots are shown. C, serum-deprived MDA-MB-231-pSuper or pSuper-sh*MLK3* was treated with 100 ng/mL HGF. D, 293T cells were cotransfected with indicated constructs, serum deprived, and treated with ± CEP-1347 followed by 100 ng/mL EGF for 30 minutes. Lysates were immunoprecipitated using HA antibody and subjected to Western blotting. E, after overnight treatment of MDA-MB-231 cells ± CEP-1347, lysates were immunoprecipitated using control IgG or FAK antibody, followed by Western blotting. Ratios of relative intensities of FAK to paxillin with control (= 1) are shown. Column, mean of 3 experiments. Bar, SE. F, immunoprecipitation and immunoblots from MDA-MB-231 cells transfected with control or Flag-*MLK3* vector. FAK-paxillin association was quantified as in E. IP, immunoprecipitation.

MLK3 silencing increases the number of focal adhesions

MLK3 modulates phosphorylation of Ser 178 and Tyr 118 of paxillin, which is required for focal adhesion disassembly (23, 24). Consistent with this, silencing of *MLK3* in BT549 cells increased focal adhesions, which were quantified as vinculin-staining focal adhesions, particularly at the cell periphery (Fig. 5A and B). Similar effects were observed using 2 different *MLK3* siRNA sequences. Silencing of *MLK3* had no effect on total vinculin protein levels (Fig. 5C). These data suggested that MLK3 is important for focal adhesion turnover.

MLK3–JNK–paxillin signaling negatively regulates Rho activity

Tyr 118 phosphorylation of paxillin leads to decreased Rho activity, enhancing focal adhesion turnover and cell migration (28). Ectopic expression of MLK3 and wild-type paxillin in 293T cells resulted in robust Ser 178 paxillin phosphorylation. As expected, no phospho-Ser 178-paxillin signal was detected upon coexpression of the phosphorylation-defective mutant, paxillin S178A with MLK3 (Supplementary Fig. S4A). In 293T cells, expressing control vector or wild-type paxillin, Tyr 118 phosphorylation of paxillin was observed.

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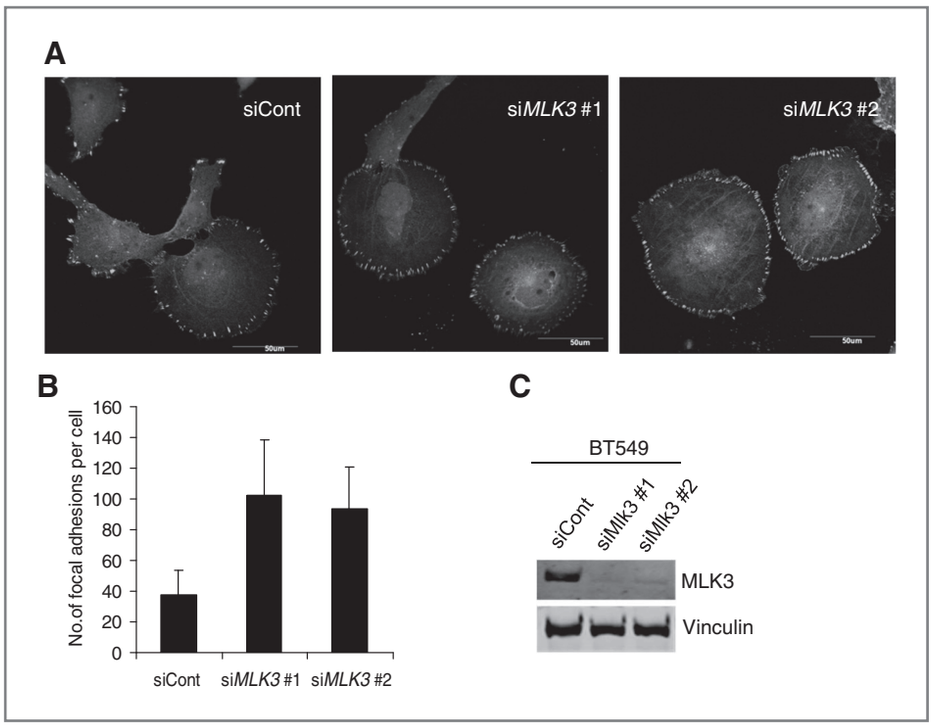

Figure 5. MLK3 knockdown increases vinculin-containing focal adhesions. A, BT549 cells were transfected with control or 2 different *MLK3* siRNAs for 48 hours. Fixed cells were stained with vinculin antibody and DAPI. Images were taken using Olympus FluoView confocal microscope. Bar, 50 μm. B, vinculin-positive focal adhesions were quantified from more than 20 cells per group using ImageJ software. Column, mean of 2 experiments. Bar, SE. C, immunoblots corresponding to A.

However, the paxillin mutant S178A was refractory to serum-induced Tyr 118 phosphorylation, suggesting that Ser178 phosphorylation of paxillin is a prerequisite to Tyr 118 phosphorylation (Supplementary Fig. S4B). Likewise, in BT549 cells, ectopically expressed wild-type paxillin, but not paxillin S178A, was phosphorylated on Tyr 118 (Fig. 6A).

Increased cellular stress fibers are observed in keratinocytes upon expression of the paxillin S178A mutant (24). As shown in Fig. 6B, a similar phenotype was observed upon silencing of *MLK3* in BT549 breast cancer cells. As Rho promotes stress fiber formation, we assessed whether interruption of MLK3 and its signaling to JNK affects Rho activity in breast cancer cells. In both MDA-MB-231 and BT549 cells, Rho activity was increased upon silencing of *MLK3* (Fig. 6C). Furthermore, inhibition of MLK3, using CEP-1347, as well as inhibition of downstream signaling to JNK with SP600125, increased Rho activity in MDA-MB-231 cells (Fig. 6D and E).

If MLK3-JNK suppresses Rho activity through Ser 178 phosphorylation of paxillin, then expression of the paxillin S178A mutant should enhance Rho activity. As shown in Fig. 6F, expression of paxillin S178A, which fails to undergo Tyr 118 phosphorylation, resulted in a marked increase of Rho activity and decreased migration of MDA-MB-231 cells (Fig. 6G). These data revealed the MLK3–JNK–paxillin signaling axis as a negative modulator of Rho activity in basal breast cancer cells.

MLK3 silencing decreases formation of lung metastases of human breast cancer cells

To determine whether silencing of *MLK3* is sufficient to prevent metastases, MDA-MB-231 cells stably expressing control vector or *MLK3* shRNA were introduced into the mammary gland of athymic nude mice. Both MDA-MB-231-control and MDA-MB-231-sh*MLK3* formed primary tumors. MDA-MB-231-control tumors were slightly larger than MDA-MB-231-sh*MLK3* tumors, but this did not reach statistical significance (Fig. 7A). Seven weeks postinoculation, primary tumors were excised. *MLK3* silencing was maintained in primary tumors over the course of the experiment as shown in immunoblots of tumor lysates (Supplementary Fig. S5).

Lung micrometastases were detected by immunohistochemistry of lung sections using a human-specific CD44 antibody. Numerous micrometastases were observed in lung sections of mice inoculated with MDA-MB-231-control cells, whereas few were found in those arising from MDA-MB-231-sh*MLK3* cells (Fig. 7B). Lung and liver tissue lysates from nude mice showed no human CD44 immunoreactivity, confirming species specificity of the antibody. Furthermore, silencing of *MLK3* in MDA-MB-231 cells had no impact on CD44 protein levels (Supplementary Fig. S6). These data provided evidence for a critical role of MLK3 in breast cancer metastasis.

Ser 178 phosphorylation of paxillin is associated with the metastatic phenotype

In this study, we have shown that prometastatic factors signal through MLK3-JNK to promote Ser 178 phosphorylation of paxillin (Fig. 7C) and migration/invasion. Using an experimental metastasis model, MDA-MB-231 cells expressing luciferase (luc2) were injected into the tail vein of nude mice. Bioluminescence imaging revealed overt metastases 12 weeks after injection. Tumor cells isolated from lung metastases (Lu) show higher paxillin phosphorylation on Ser 178 compared with parental MDA-MB-231 cells (Pa), both basally as well as in response to either CXCL12 or HGF (Fig. 7D). Furthermore, CEP-1347 inhibits both CXCL12- and HGF-induced Ser 178

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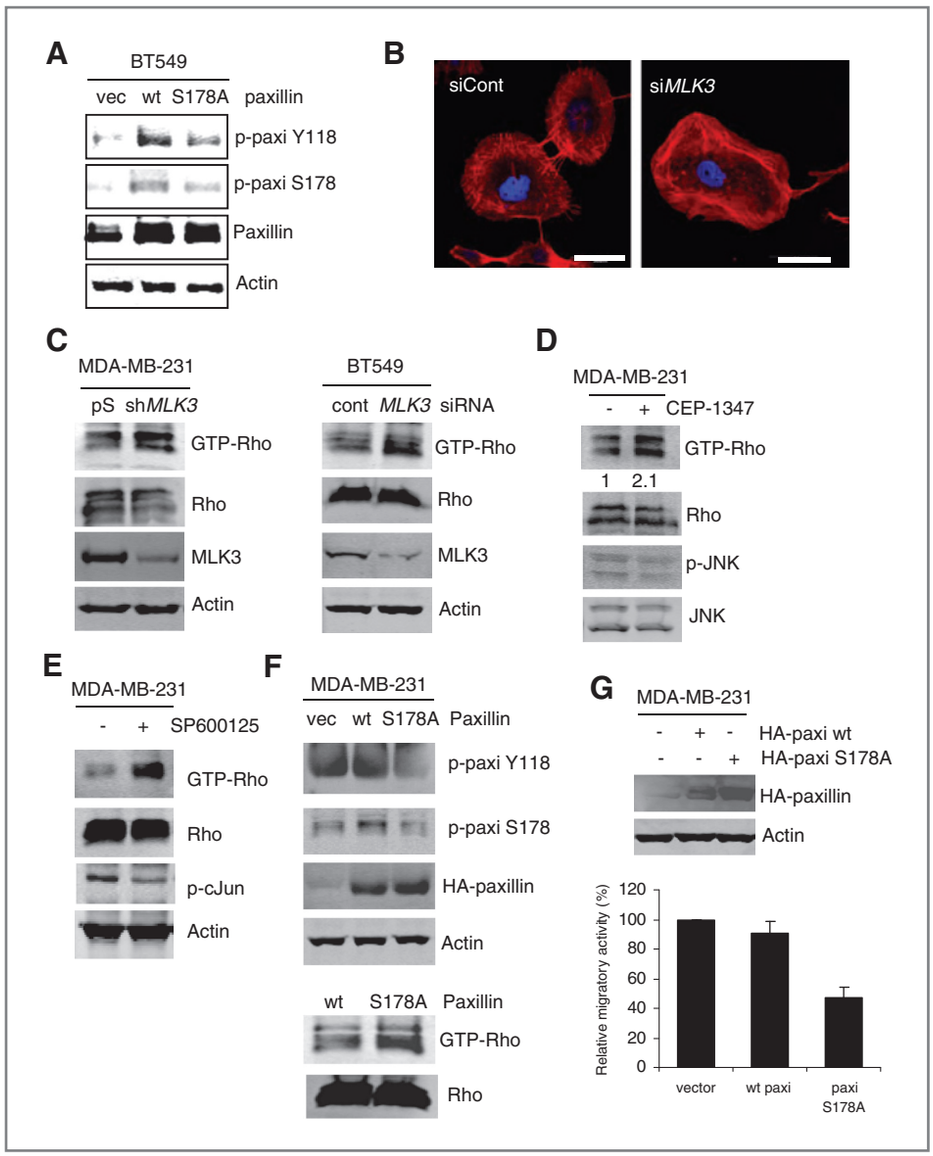

Figure 6. MLK3-JNK–mediated Ser 178 phosphorylation of paxillin is necessary for Tyr 118 phosphorylation of paxillin and inhibits Rho activity. A, immunoblots from BT549 cells transiently expressing HA-paxillin wt or HA-paxillin S178A mutant after serum deprivation and treatment with 10% serum for 30 minutes. B, BT549 cells were treated with control or *MLK3* siRNA for 48 hours, stained with phalloidin (F-actin) and DAPI (nucleus) and imaged using confocal microscopy. Bar, 50 μm. C, immunoblots from Rhotekin pulldown assay of MDA-MB-231-pSuper or MDA-MB-231-pSuper-sh*MLK3* (left) and BT549 cells treated with control or *MLK3* siRNA (right). D, immunoblots from Rhotekin pulldown assay of MDA-MB-231 cells treated with ± CEP-1347. E, immunoblots from Rhotekin pulldown assay of MDA-MB-231 cells treated with ± SP600125 for 6 hours. F, Rhotekin pulldown assay and immunoblots from MDA-MB-231 cells expressing HA-paxillin (wt) or HA-paxillin S178A mutant. G, transwell migration assay of cells from F was carried out with corresponding immunoblots shown.

phosphorylation of paxillin in Lu cells (Fig. 7E), indicating these cells are still sensitive to an MLK inhibitor.

Screening of a panel of human mammary epithelial and breast cancer cell lines revealed a correlation between p-Ser 178 paxillin and metastatic potential (Supplementary Fig. S7). These data, taken together, suggested that phosphorylation of paxillin on Ser 178 may be a predictor of lung metastatic potential.

Discussion

Deciphering key signaling pathways underlying breast cancer cell migration and invasion may reveal novel therapeutic targets for effectively treating or preventing metastatic breast cancer. We previously showed MLK3-JNK signaling upregulates multiple AP-1–driven invasion genes and promotes a malignant phenotype in mammary epithelial cells (16). JNK is important in breast cancer cell migration and invasion and breast cancer progression (24, 33, 34). Yet, how MLK3-JNK signaling regulates cell migration machinery remains largely unknown.

In this study, we report, for the first time, that in response to CXCL12 and HGF, MLK3 signals to JNK to control phosphorylation of paxillin on both Ser 178 and Tyr 118 (Figs. 2–4), phosphorylation events that are essential in cell migration (24, 35). MLK3 modulates interactions between 2 key focal adhesion proteins, paxillin and FAK (Fig. 4). MLK3–JNK–paxillin signaling negatively regulates Rho activity to promote focal adhesion turnover in cell migration (Figs. 5 and 6). Finally, MLK3 is critical for formation of breast cancer lung micrometastases in a mouse xenograft model (Fig. 7). The importance of paxillin phosphorylation is highlighted by the finding that cells derived from MDA-MB-231 lung metastases show higher phosphorylation of paxillin at Ser 178, compared with parental MDA-MB-231 cells, implicating this phosphorylation site in breast cancer metastasis. Taken altogether, our data

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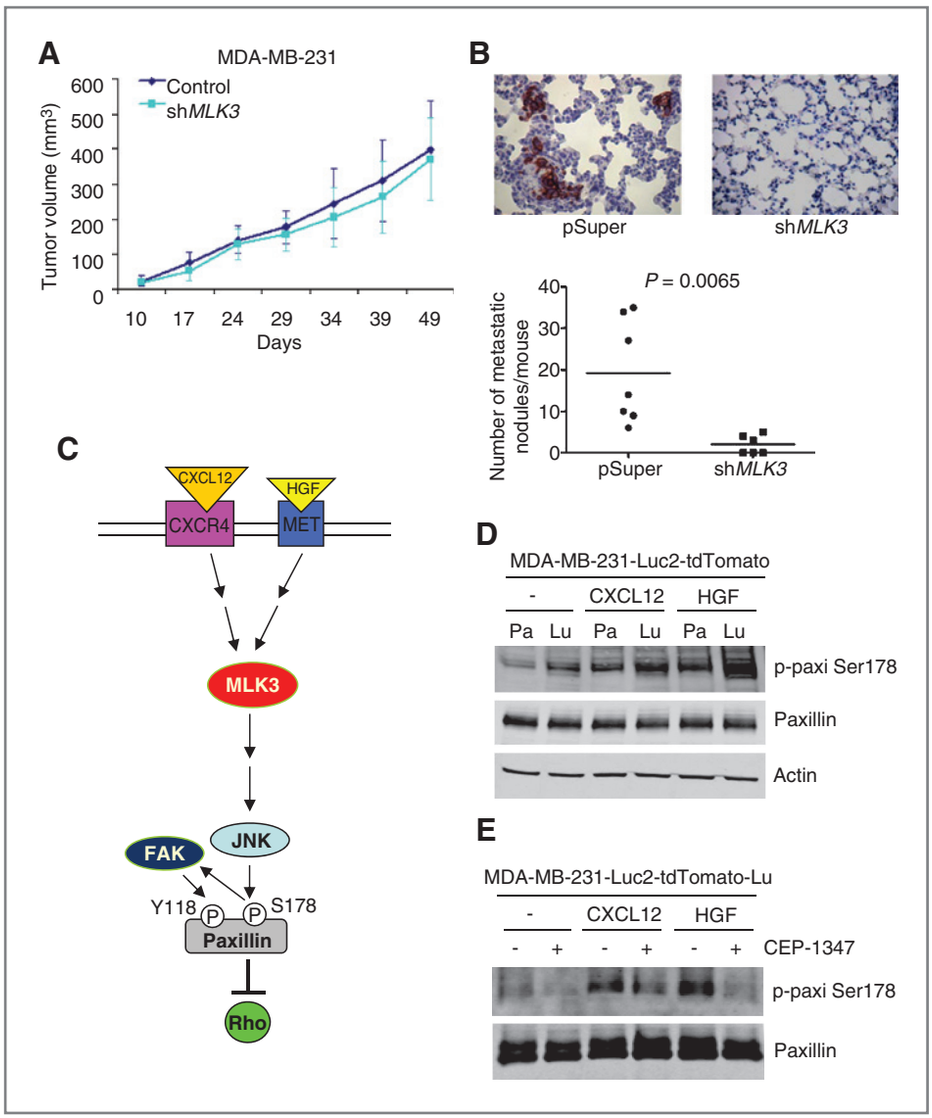

Figure 7. Depletion of MLK3 prevents formation of lung metastases. A, tumor growth curve of MDA-MB-231-pSuper or MDA-MB-231-pSuper-sh*MLK3* inoculated into mouse mammary fat pads. B, immunohistochemistry of lung sections using a human-specific CD44 antibody. Magnification, ×400. CD44-positive nodules were quantified in 10 sections per mouse. Statistical analysis was done using GraphPad Prism 5. C, schematic model showing MLK3-JNK-pSer 178 paxillin signaling axis, activated through prometastatic factors CXCL12 and HGF, leading to FAK-mediated Tyr118 phosphorylation of paxillin, and suppression of Rho activity. D, cultured cells derived from lung metastases of MDA-MB-231-Luc2-tdTomato were designated as Lu. Immunoblots from serum-deprived parental MDA-MB-231-Luc2-tdTomato cells (designated as Pa) and Lu cells treated with CXCL12 or HGF for 20 minutes. E, serum-deprived Lu cells treated with ± CEP-1347 overnight, and treated with CXCL12 or HGF as in D.

reveal a novel MLK3–JNK–paxillin signaling pathway that regulates breast cancer cell migration and invasion.

Paxillin undergoes dynamic phosphorylation during cell migration (21). Ser 178 phosphorylation of paxillin is essential for cell migration (24). We show both CXCL12 and HGF signal through MLK3 to paxillin, consistent with our data showing that MLK3 is required for CXCL12-induced breast cancer cell migration (Figs. 1–3). Thus, MLK3 emerges as an important signaling node that relays extracellular cues to JNK to control paxillin phosphorylation. Although it is possible that other MAPKKKs contribute to paxillin phosphorylation through JNK, MLK3 seems to play a dominant role, at least in basal breast cancer cells. Because CXCL12 and HGF are consistently linked with invasion and metastasis, our findings provide a strong rationale for targeting MLK3 in the context of breast cancer metastasis. In agreement with our findings, localized JNK activation and Ser 178 phosphorylation of paxillin is observed during migration of rat kidney epithelial cells, which involves the aPKC-Exocyst complex (36). Interestingly, PKC is important for activation of MLK3 in response to free fatty acids (37). Whether PKC plays a role in CXCL12- or HGF-induced MLK3 activation remains to be determined.

Rapid assembly and disassembly of focal adhesions is a well-described property of many migrating cancer cells. Experimental disruption of focal adhesion turnover typically results in migratory defects in cancer cells (38). Phosphorylation of paxillin at Ser 178 by JNK (24) and Tyr 118 by FAK/Src (39, 40) is critical for focal adhesion turnover and cell migration (24, 35, 41, 42). For instance, a tyrosine phosphomimetic mutant of paxillin enhances focal adhesion turnover, whereas a non-phosphorylatable mutant shows defective focal adhesion turnover and migration (22, 23). Furthermore, phosphorylation of Tyr 118 on paxillin is implicated in cancer invasion and metastasis (43). Our results support a model in which MLK3 is required for focal adhesion turnover in cell migration through controlling Tyr 118 phosphorylation of paxillin (Fig. 4). Indeed,

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MLK3 silencing increases the number of focal adhesions in breast cancer cells (Fig. 5). Furthermore, experiments using a nocodazole-based assay (44), in which nocodazole washout promotes microtubule formation and focal adhesion turnover, reveal a defect in focal adhesion disassembly upon MLK3 silencing in MDA-MB-231 cells (data not shown), supporting the necessity of MLK3 in focal adhesion turnover.

Elevated levels and activity of FAK are found in high-grade human cancers, including breast cancer, and correlate with invasive phenotypes, metastatic disease, and poor prognosis (45). FAK inhibitors are currently in clinical trials for treating human solid tumors (46). Activated FAK recruits Src to form an active FAK/Src complex. Our data showing that MLK3 promotes interaction of FAK with paxillin may explain how MLK3 controls Tyr 118 phosphorylation of paxillin and promotes focal adhesion turnover, because association of FAK with paxillin promotes tyrosine phosphorylation of paxillin and is correlated with less stable focal adhesions (47). Ablation of the Ser 178 phosphorylation site on paxillin decreased phosphorylation of Tyr 118 (Fig. 6), suggesting that, at least in this experimental context, Ser 178 phosphorylation is a prerequisite for Tyr 118 phosphorylation. This is in agreement with the finding that paxillin S178A has decreased affinity for FAK (32). In our working model, MLK3-JNK-Ser 178 paxillin phosphorylation regulates association of FAK with paxillin and indirectly controls subsequent tyrosine phosphorylation of paxillin (Fig. 7C).

Focal adhesion dynamics are tightly controlled by Rho GTPases (20, 25). Active Rho increases stress fibers and focal adhesion maturation and decreases focal adhesion turnover (48). Although Rho activity is required for cell migration, aberrantly high Rho activity also impairs cell migration (26). FAK promotes focal adhesion turnover, in part, through suppression of Rho activity (48). In particular, FAK/Src-mediated Tyr 118 phosphorylation of paxillin has been proposed to release p190 Rho-GAP from its sequestration with Ras-GAP, leading to downregulation of Rho activity (28). Our experimental evidence supports a model in which the MLK3–JNK–paxillin Ser 178 signaling axis negatively regulates Rho activity, through FAK-mediated tyrosine phosphorylation of paxillin. Indeed, *MLK3* silencing in breast cancer cells resulted in increased focal adhesions (Fig. 5) and stress fibers (Fig. 6), 2 Rho-associated phenotypes. In A549 lung carcinoma cells, a role for MLK3 in limiting Rho activity through an interaction with p63 RhoGEF has also been described (17). In summary, we have identified in basal breast cancer cells, a distinct pathway involving an active MLK3–JNK–paxillin axis that functions to negatively control Rho activity.

In a xenograft model in which MDA-MB-231 cells were introduced into mammary fat pad of nude mice (49), MLK3 silencing inhibited spontaneous lung micrometastases (Fig. 7), in agreement with findings that MLK3 knockdown reduced lymph node metastases of MDA-MB-231 cells (50). Although MLK3 has been shown to promote cell survival (16, 50), which might contribute to formation of metastases, we propose that an important mechanism through which MLK3 promotes metastasis is through facilitating cancer cell migration and invasion.

Our study shows that MLK3 controls Ser 178 phosphorylation of paxillin, which is required for cell migration. Interestingly, phospho-Ser 178 paxillin correlates with metastatic potential of breast cancer cells, suggesting that phospho-Ser 178 paxillin might be a predictive biomarker for metastasis. Our novel findings show that prometastatic factors found in the tumor microenvironment converge on MLK3 to promote breast cancer cell migration and invasion. Due to the fact that the MLK inhibitor CEP-1347 efficaciously blocks invasion in response to such factors, we are currently testing the effect of this compound in a preclinical study using a mouse xenograft model. Our findings indicate that MLK3 regulates phosphorylation of paxillin and its interaction with FAK. We also provide evidence that the MLK3–JNK–paxillin axis negatively regulates Rho activity and focal adhesion turnover. Finally, we show a critical role of MLK3 in breast cancer metastasis. Thus, targeting MLK3 could be a promising therapeutic strategy for treatment or prevention of metastatic disease in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Chen, K.A. Gallo
Development of methodology: J. Chen, K.A. Gallo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Chen, K.A. Gallo
Writing, review, and/or revision of the manuscript: J. Chen, K.A. Gallo
Study supervision: K.A. Gallo

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